Mutations and Human Health

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Module Synopsis

The module provides an advanced understanding of the effects of carcinogenic agents on human health, and develops skills in investigating and assessing DNA damage caused by genotoxic compounds to improve the prevention and treatment of cancer and human disease.

Learning Outcomes

Demonstrate a critical understanding of carcinogenic compounds, and their uses and existence in a historical context.

Demonstrate a critical understanding of the mode of action of DNA damaging agents with respect to their carcinogenicity, and be able to critically discuss exposure through to cancer.
Evaluate genetic toxicological data in a qualitative and quantitative manner.
Demonstrate critical knowledge of the current genotoxic compounds in the public eye, and be able to have an unbiased view on the potential carcinogenicity of these agents.
Implicate genetic toxicology methodology to assess nano-safety and show a critical understanding of the development and uses of nano-materials and their impact on human health for risk assessment.

Chapter 1 (Mutations of human health)

This chapter contains

- Introduction to genetic toxicology

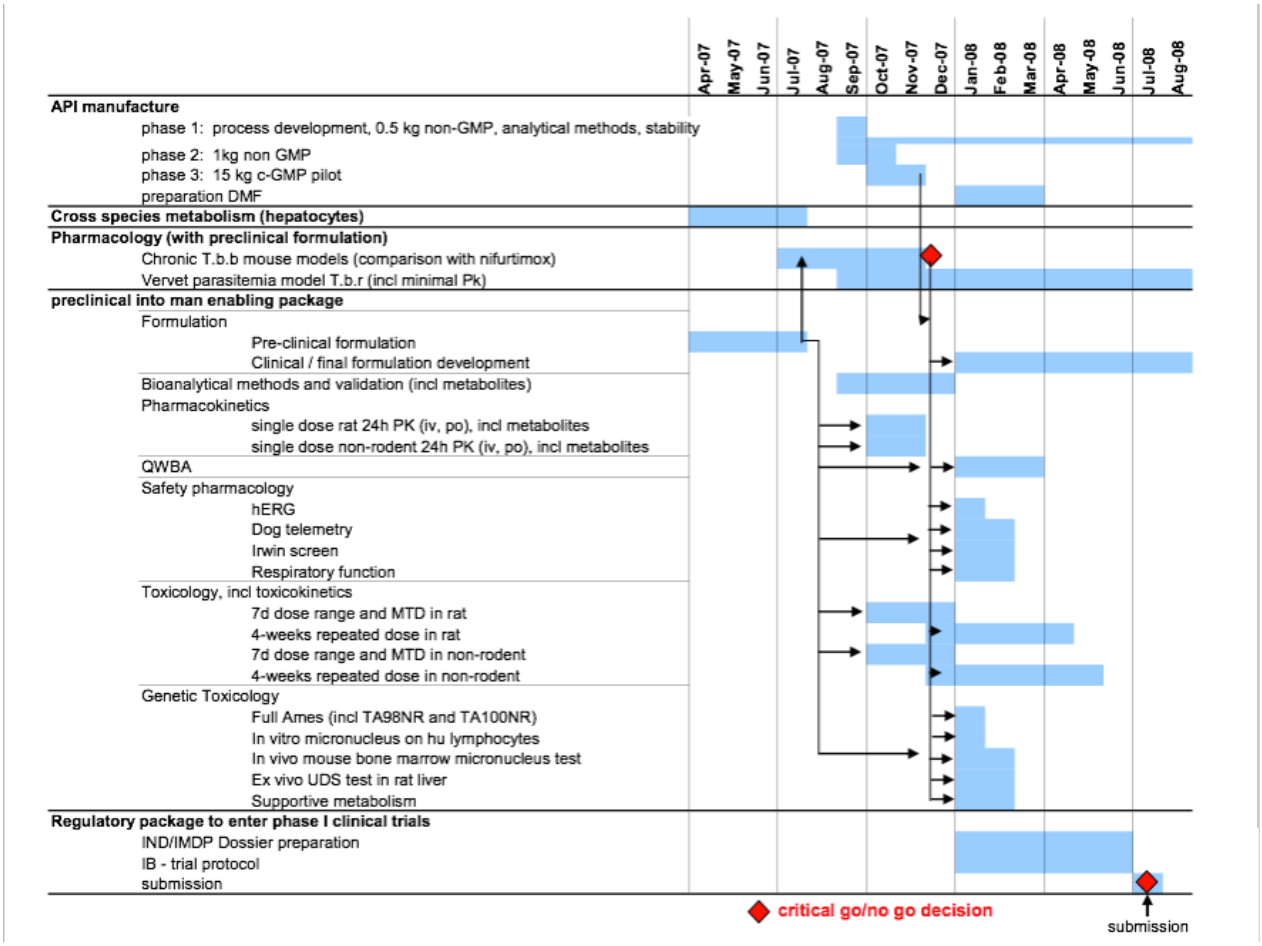
Introduction to genetic toxicology

So this topic is about mutations of human health and we are going to be covering these different angles seen below.

- History of Genotoxic agents and how they can lead to cancer. **GEJ**
- Assays with OECD guidelines and potential techniques for the future. **DK**
- Mutations. **GEJ**
- Endogenous and Exogenous DNA damage. **GEJ**
- Nano-safety: nano-materials health impact and risk assessment. **NS**
- Genetic Toxicology in the pharmaceutical industry. **AL**
- Toxiocogenomics / Novel treatments for Neglected Diseases. **DT**

Its more of an industry focused moduel and how chemicals that we are exposed to can be assessed and sort of commercialised. We are looking at substances in the pharmaceutical industry and agerochemical industry and we are trying to figure out and prove that there safe and if they are not going to increase our genetic burden then there not going to give us cancer. So to get to that stage are going to have a background of genotoxicity and how it is induced. We then have professor david kurkland coming in talking about the different genetic toxicology assays they use in industry in both in vivo and in vitro and who you do and what you do with the results. It is very useful information and it is used all the time. We will look at some information and how there induced by these substances and how they are produced over time endogenously and exogenously and we are going to look at some examples of how these chemical and how we are exposed to these chemicals both endogenously and exogenously so just inner own cellular metabolism and how we are exposed to loads of nasty stuff as well and why they dont kill us. So nano-safety we will talk a bit about nano toxicology which has quite a big group her ein swansea and we have some of the leading experts here at swansea. Then we will look at some nice case examples to give assume idea of what this type of science is all about and then we will have some working examples for GSK from anthony who will also detail how even if you get a positive of one of these tests. Are you going to bin drug thats going to make a billion pounds a year or are you going to figure a way of showing that its not safe for certain reasons. Then toxicogenomics are quite basic assays that looks at do they cause mutations or not have they got chromosome damage or not and there are some novel ways that toxicogenomics where you can look at gene expression profiles so you get some cells or an animal and treat it with a certain chemical. If you get a chang in gene expression then can you link that to risk as well. So thats for toxicogenomics and lots of people think that thats a good thing and also a bad thing as well. Thats from professor david tweets who is also going to talk about one of his consultancy projects

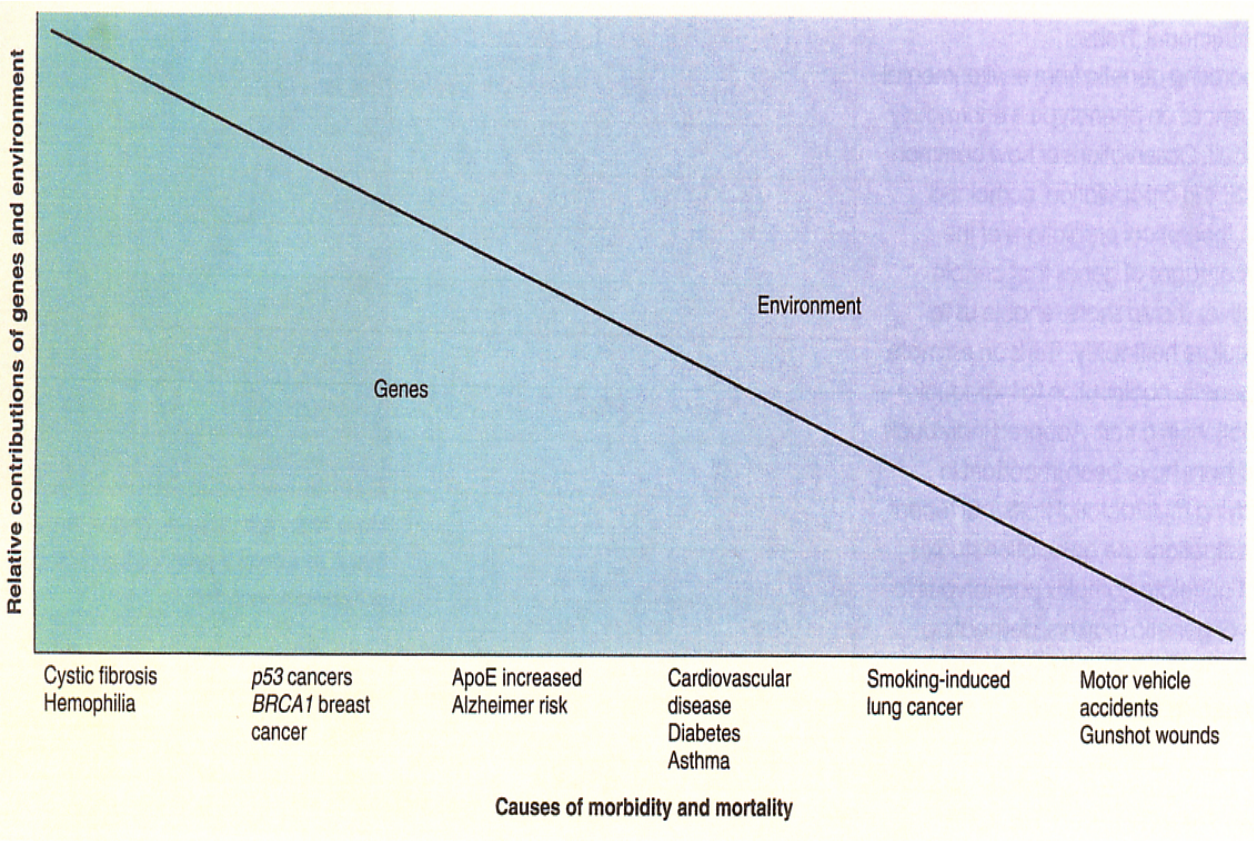
where he worked with the drugs and disease incitive, where he looked at some guys worked that got fined in the 70s because it got flagged up in an in vitro microbial gene mutation test as being a mutagen then they went back to and realised it was only bacterial specific mutagen and realised it was wrongg and published papers on it and now its in stage 2 or stage 3 clinicl trials and it has the potensial to save about 20,000,000 peoples lives from sleeping sickness and chagas disease as well. So a nice case example about how this paper is actualised in the real life situation so again we have more of an industry focus in this modele and the cancer module is more sort of clincally focused with people form the NHS and such coming in. So heres a nice confusing thing for you that highlights where genetic toxicology comes in. So that is the actual profile from that drug which is actually showing that they carried out an assessment over a time period with this drug called fexinadazol for river blindness thats funded by the gates foundation. We dont care about the drug to much at this stage we just really wanted to see that this is the kind of thing you do. You get a drug, you dont want to carry



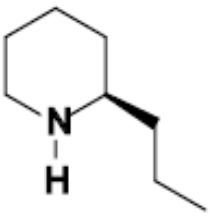
them out all at the same time. So if your going ot market this drug and again its going to be a blockbuster drug giving you around a billion pounds a year. You don't want to stager the safety testing because the longer it takes to get it out the more millions you loose. So the idea is that you do all of these things at the same time and at the end of it all you want is a safety profile to have no flags and showing that everything is noice and safe and that the efficacy shows that it actually works and then you can go on to the stage 1 human trials then. So what we are doing here then is looking at the month by

month action protocol on how you actually make this stuff in big amounts. The pharmacology side starts going towards the formulation and how to make this stuff to be distributed throughout the system. pharmacogenetics so dose it get distributed around the system quite nicely. Then the safety side is where we come in, in Swansea we dont have an animal house we just do the in vitro side of things. Obviously these tests if its a pharmaceutical substance then you need to carry out in vivo assessment and show that its safe in these animals as well. So here we have things like safety pharmacology, standardd toxicology and these things look to se if these compounds are refining the organs of the animals and then genetic toxicologyy comes in and just wants to know really are these substances causing gene mutation. Are these substances causing chromsome brakes, are they causing segregation of mitosis causing a loss or gain in the chromosomes. So0 thats what we are doing here and thats just to show us how it lines up with everything else. Then we get all of that we wrap it up together and give it to people like the FDA and they say ok go ahead or they say no and bin the work.

So we are going to look at the history of it all now and look at how genetic toxicology links in to all of this. We will mainly be talking about pharmaceitcal industries but we will elude to some other industries as well like the agrochemical, the petrochemicals and finally the food flavours industry. So what we need to think about is say we we have conditions on the left side of the diagram which is entirely linked to your genetics like the diseases cystic fibrosis and heamophilia. We get over towards this side where the environment starts having more of an impact. This side of things where smoking is quite a nice example. Smoking indues lung cancer where you have some pre disposition to getting increased levels of cancers. You have some strange oncogenes and some strange tumor suppressor genes. But mostly it has been



induced by the heavy amounts of smoking and nasty things like polyarmoatic hydrocarbons and again we will talk about those later on. So our idea is trying to figure out how the environment can impact on the genes really. So why did all of this start out, it started out with an interest in the underlying mechanisms of mutagenesis and realising that radiation and chemicals ar actually doing some strangee things. you get some cells and start playing around with them and you realies that radiation and chemicals start killing the cells and starting to make them divide in strange ways. Then later on in the world wars it was the era of the atomic bombs that had some sissues that were causing people to get cancer and they started thinking about what the mechanisms of this could be. There is mustard gas as well where they saw it as a blistering agent in the world wars. All these blistering agents do is enter the body and attache to parts of you DNA and then inhibit the cell from dividing. So those the cells die and then they lyse (necorsis) and this causes a blister. These sorts of thing shave some side effects as well. So because people wanted to get an understanding if this they started getting a bit worried as they are being exposed to many more agents than was previously thought which is when regulations were starting to get introduced. So the identification of the role of these mutations in cancer induction of progression. So people started understanding that these nasty chemicals and substances caused cell death and cancer and then people started getting appreciation of why people were dining from cancer. So a bloke seen here below is Philippus Aureolus Theophrastus Bombastus von Hohenheim said that everything is poison and there is nothing that we know of that is without poison, it is only the dose that can commit something to be considered not poisoness. This comes up quite a bit and even recently where oyu here in the press where you here every week saying that something gives you cancer and then the next it docent give you cancer. This is really all about the concentration and all about the system people are testing it in. So even knower days people are still arguing about this sort of thing where they realise that if someone is exposed to a nasty chemical like a bottle of coke then there is loads of compounds that make up the coke and the bottle that if we broke down we would find are carcinogenn but because there at such low levels they don't give us an effect. So you can scare people but really you have to have some realistic understanding to realise that the concentration is really the important factor. This is not so much genetic toxicology and is more toxicology and even before christ so BC people were using chemicals to kill each other as depicted in the diagram below which is the killing of Socrates by Hemlock.

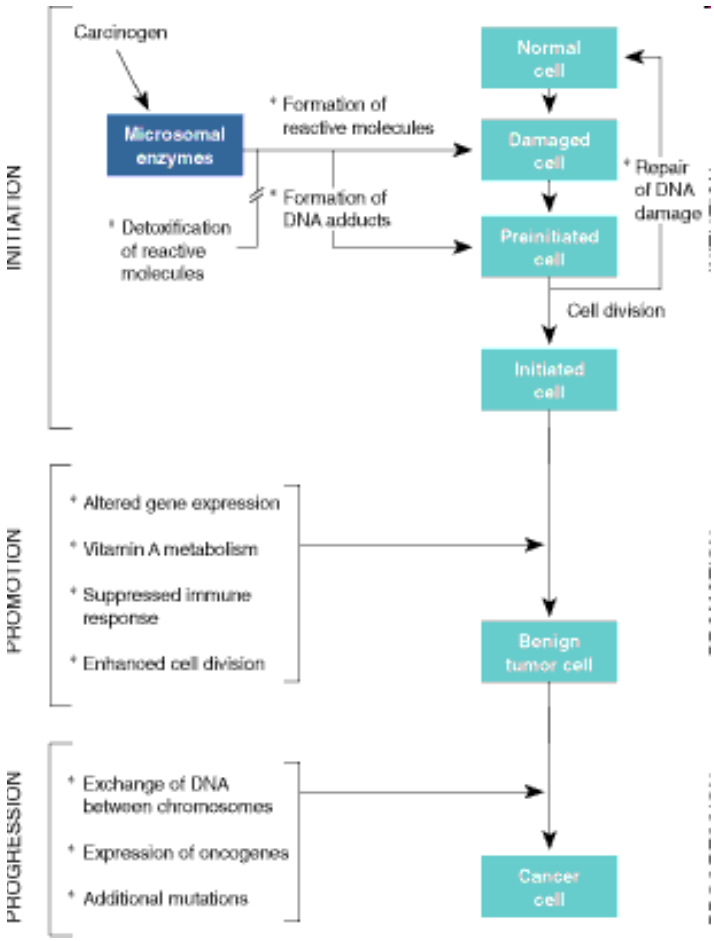


this was done with an alkaloid called coniine which could cause paralysis, convulsions and eventually death. So this would lock open the synaptic gates cause a paralysis and that was that. So even back in these old days people had an understanding of what the substances were and people also had an understanding of what cancers were, which we will see in a minute. So even the dinosuarse were said to have types of cancers where there bones show evidence of cancers. The Egyptian mummies were shown to have one cancer also. So you see that cancer is prevalent throughout history and the only thing thats prevalent about the eygypcians is that hey started getting a bit of an understanding of some types of cancers. So in Egypt there is evidence for 8 differt breast tutors and ulcers and they actually came out of a depictn that was drawn on a papyrus. So they came in with a looped bit of metal and they would cut off a bit of tissue. They would see that there is some sort of tumour in these breasts, heated up a bit of metal and chopped it out and treated cancer even before in these early days. So there has a always been this big understanding of cancer and the understanding that if you chop it out then thats a good thing.

So the word cancer comes from Hippocrates who named these tumours as carcinomas or as carcinos because they resembled a crab with these spreading out region. We learn more about that pattern in a different module but really its due to over vascularisation or these tumours. So back in 15 hundreds people started chopping each other up and seeing a better understading about cancers and then with the invention of the microscopes they saw at the cellular level the changes in these types of cells. So they started doing pathology and seeing that in advanced cancers the cells are completely changing there morphology from one cell type to another. These are the sort of advancements you need these things like microscopy. Later on they stared linking occupational exposures to certain types of cancers as well, so we got occupational cancers showing that a high incidence of breast cancer was seen among nuns. So because nuns weren't actually sexually active and didn't have children then they didn't have these periods in there lives where there menstrual cycles were stopped and they didn't have these changes in the hormone levels. Thats been linked to the increased levels of breast cancer in the nun population. There are also nice paper on this in the lancet. Later on all the substances that Swansea is more associated with are Percivall Potts showed that in a certain type of industry people were getting certain types of cancers were these chimney sweeps. So there exposed to thes high levels of poly aromatic hydrocarbons. If you burn something like a cigarette or coal or whatever then you get these poly aromatic hydrocarbons. These people up a chimney all day would ingest this stuff and they were getting increased levels of this type of cancer. The Percivall Potts being the earliest one charecterised. Later on people started getting bit more obsessed with fancy dies and these azo-dies which are things that are used in cloths and used in lots of products to make them colourful. These Azo-dyes were actually giving cancer as well. So lots of types of cancer but these are a few examples. They started linking in these working industries to getting increased levels of cancer because of there increased exposure to Azo-dyes. So a lot of the time again in the press you may here about increased risks and things like this and its not normally the actual

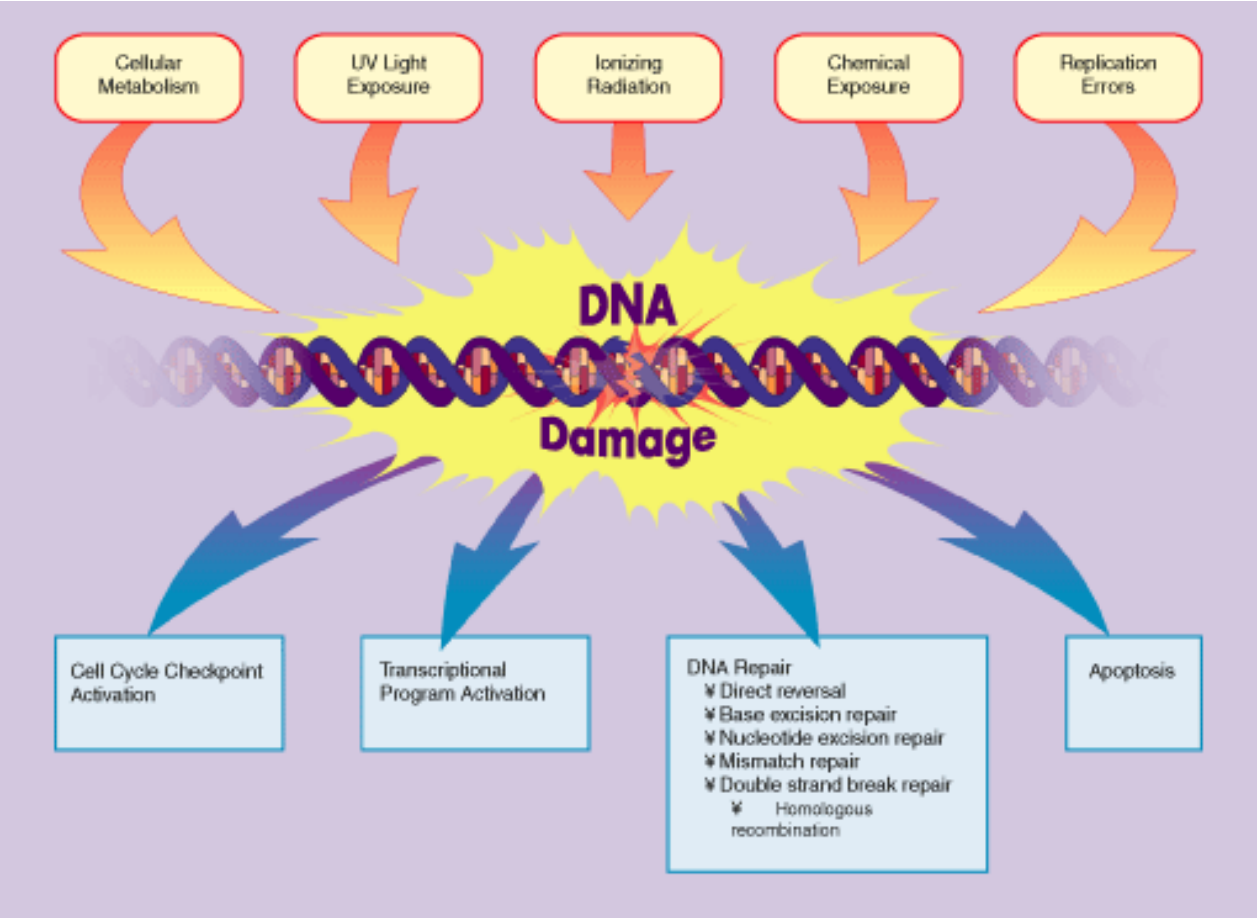
population thats at risk its the people in the work houses that are being exposed to the high levels that should really be worried about the whole thing. So later on we started seeing these things like coal tar, people used to treat exa with coal tar and this has got insanely nasty stuff in and if your rubbing these things on to your skin then you are getting these poly aromatic hydrocarbons in your skin. Treating animals with this and rubbing it on animals actually gives them skin cancers. Then they stared getting so interested in it they actually synthesised Benzo-a-pyrene which is a super carcinogens that is in cigarette smoke and then when you synthesis it your not testing the big mixture of everything your testing just that one chemical you can get a really good idea of whats going on when you can synthesis it and get a really good idea about whats going on when you can synthesis it and test it specifically. Then the whole field started to develop and they started realising that if you got model animals and started treatng them with nasty stuff then you could actually induce things like cancer. So we are not going to go into a lot of depth about what cancer is but it is useful for this lecture.

So its the uncontrolled multiplication of cells so what we are thinking about is chemicals which lead to this uncontrolled multiplication, lead to mutations in proto-oncogens and tumor suppressor genes which are going to make your cells divided in a strange way and the terms we use are things like benign and where a cancerous cell is contained in one place and malignant when it gets worse and they can spread to other areas and cause things like metastasis. So cancer in humans, the reason why we are going to be talking about these different terms is because you can have substances which are cancer initiators and cause and initiation of cancer. So these are like the first mutation in a certain gene, promotion where theres an accumulation of these diffenret mutations in the different genes. Progression where there the cells are actually changing into different types of cells and malignancies when they start to migrate. So how we asses this in in vitro and in vivo well we can use cell lines, transform cells and the easyest way of seeing this is dose this substance cause a tumor in the experimental animal and what we are talking about in this module is other assays so you don't have to carry out these assessments in animals. which is good because the bioassay requires 400-800 animals and takes two years to compleat. Genetic toxicology takes no animals and much less time so these are just some of the benifits. So we will be talking about some of these things. So what causes cancer basically every thing causes cancer. Organic chemicals things like asbestos, there are hormones as well like 17beta estradiol so even our own hormones which we produce endogenously can cause us cancer as well. With hormones there are lots of substances that



can mimick these hormones which we said can have deleterious effects. Then micktures, which is quite hard to test but then mixtures of things can give you cancer. too.

So hormones are quite a bid groups that we will be going back to throughout this module some inherited conditopnas and really we will be talking a lot about reactive oxygen species. So these are thing like the hydroxyl radical and these can be induced by many different things like increased stress and lots of iratation. Lots of the antioxiDNAts try to sell you are oxygen scavengereswhich can just come along and soak up these reactive oxygen species. One of the main deleateriouse effects are from ionising radiation and it causes reactive oxygen species in close proximity to your DNA. So theres water next to your DNA and ionizing radiation cause these to turn in to the hydroxyl radicals and this causes you to get some DNA damage but again we will get some information about this later on. We have also got some external factors as well so we have our habits like smoking and the diet, so your own lifestyle can mean your exposed to higher levels of these certain types of chemicals. Viruses as well can come in and can cause DNA drage and get in between the bases in you genes. chemicals and radiation. So basically we are exposed to a bunch of diffenret things and were not all dead. So there is a lot of preventative measures to. So if we go along the top of the diagram here things like cellular metabolism where just in the mitochondria we metabolism chemicals and when you metabolise something your likely to get some sort of reactive byproducts. UV light exposure is linked to cancers things like cyclobutane pyrimidine dimers they come in and make two bases join together just through photo reactivation. Ionisingg radiation we will talk about a bit, even in this lecture but mainly chemical exposure because we are talong about the



pharmaceutical industry they are making chemicals and they want to make sure that its safe. So thats what we will talk about quite a lot. The replication errors so when the DNA is trying to duplicate itself, you can actually mess this up and cause some mage just endogenously. But as we said we are not all dead we haven't all got cancer because there are some nice mechanisms to make sure we don't get cancer. So things like cell cycle checkpoint activation, if you have got some DNA damage it gets picked up in the cell cycle checkpointt, it stops at the cell cycle checkpoint and give the cell some time to repair that damage or giving the cell time to induce apoptosis which is the programd cell death. So in this case if we are talking about cancer in this case it is a good thing because we are not going to produce a daughterr cell with the increased damage,.So cell death to cancer is actually a good thing because it is preventing it. Then shen you have this damage you can activate some different programs as well. The up regulation of certain genes and pathways with DNA repair being one of the main ones. If you have a substance like a reactive oxygen species like a bento-a-pyrene di epoxide interacting with the DNA. If its pickd up then we have some very niceness and tidy repair mechanisms that have evolved naturally that can get that DNA adduct and remove it in a clever way. So we will look t some examples now and also some temrinology. So these terms will be used throughout the module to keep note of them really:

Genotoxicity = these are really the studies of the chemicals that causes toxicity to your genes. That is the all encompassing term for everything else written below.

Mutagenicity = More specifically the things things that cause a gene mutation or some kind of base pari change or causes a deletion or just one base pair to become another base is mutagenicity caused by mutagens.

Clastogenicty = This disruption of a chromosome changes or some thing that causes chromosome brakes is called clastogenicty.

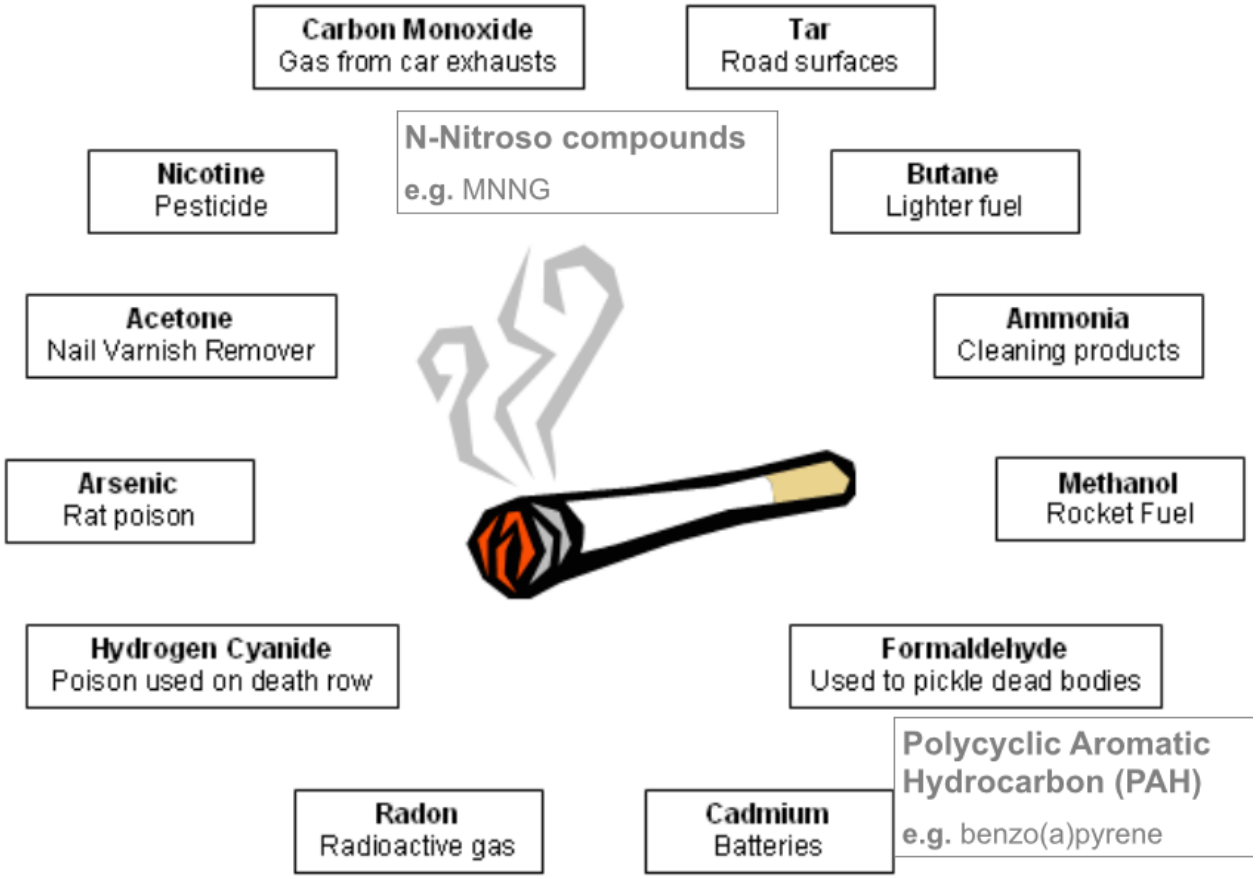
Aneuploidy = being linked to down syndrome is basically a change in the number of chromosomes so something that can induce this would be called an anuegen causing aneuploidy. So this is education of numerical chromosome changes such as the gain or loss ozone chromasome or the gain or loss of multiple chromasomes.

So one of the early mistakes and for which products are so heavily regulated nowadays that were made were the spreading of DDT or dichloro-Diphenyl-Trichloroethan. This wasted as a pesticide to try and fight the vectors of malaria so it actually killed off insects and particular in these areas of africa which carried these different types of disease. A pesticide for vectors of malaria, typhus, yellow fever and sleeping sickness.DDT was really quite scuffle in killing of these insects in quite a good way. the problem is that it had some side effects particularly when tested in vitro and in vivo where it was seen to be a probable human carcinogen and seen to be an endcrine disrupter as well. So it actually mimicked hormones it was affecting the estradiol receptor and the androgen receptor. So this chemical that they were using to kill off all these insects were actually coming in and interacting with these receptors and the thing is here that these are hormone receptors and they are also transcription factors and they regulate

absolutely loads of genes. So if you start messing around with these then you are going to have loads of effects. So this was back in the seventies, these were really quite massive treatment programs were people were being exposed to quite high levels of DDT. Then they started testing this quite heavily and

This machine is spreading a kind of fog of DDT spray to see if it will kill the mosquitoes and other insects on the beach. Outdoors, the spray soon thins out and does not harm people.

saw that the animals systems cause liver cell tumors and its causing liver carcinomas and hepatic sarcomas and lung carcinomas. So there is really a conflict nowadays where they are saying that they didn't see a huge increase in human cancers in these areas and this is probably due to the levels or the expression levels. We wont go into to much detail there we will just realies that this is one of the main reasons that chemicals and compounds are so heavily regulated at the moment. Because you get problems like this and realise that everyone is being exposed to these nasty chemicals and this si the type of ting thats happening and even beaches in claifornia. where they would go up an down the beaches and just spray this in people faces. This made every oen happy because althoe flies are dining but when you see that it causes liver cancer and it messes up your endocrine pathways its not really something that you want to be spraying yourself or your kids with. Something else we quite happilyy expose ourselves too a lot of the time are cigarettes and the byproducts of burning things like these N-Nitroso compounds and all these poly aromatic hydrocarbons seen below. We have got things like bento-a-pyrene which we keep going back too. Obviously there are al of these other things but the main one we will be looking at is the main one thats been associated with the lung cancers and in the cigarette smoke are these poly aromatic hydrocarbons. So because of this we have got a quite good understanding of human



experiments reall. In do these chemicals cause cancer, yes they do and we can test these peoples tissues for deleting the levels in these tissues we can actually detect the types of DNA adducts that are being caused and we can see someones lungs cancer then we can see specific elements or adducts produced by the substance in the lung cancer and we can see that this substance is inducing lung cancer. Theres auto mutation profiles as well so a very clever way linking that types of substance to those increases in cancer a nice thing here is when we mix our drinks in with coffee, a lot of these compounds are in coffee as well but again a very low concentrations. On the levels within coffee as well we have bento-a-pyrene, hydrogen peroxide, formaldehyde, benzene. This is in coffee which is a product that we drink quite a lot. If this was a new product then it would be band strait away but because of its industry and history then it cant really be banned. So we realise that we are exposed to all these different things on purpose and the also because we don't really know what were ingesting as well. So because of that we are exposed to these different things its mostly environmental factors in cancer deaths, diet is the biggest cause of cancer and especially because of the increased levels of estradiol because estradiol is made from the afatty substance that are being increasingly ingested. So with an unhealthy diet you get

APPENDIX TABLE III

Carcinogenicity in Rodents of Natural Chemicals in Roasted Coffee[88]

Positive:	
N=19	acetaldehyde, benzaldehyde, benzene, benzofuran, benzo(*a*)pyrene, caffeic acid, catechol, 1,2,5,6-dibenzanthracene, ethanol, ethylbenzene, formaldehyde, furan, furfural, hydrogen peroxide, hydroquinone, limonene, styrene, toluene, xylene
Not positive:	
N=8	acrolein, biphenyl, choline, eugenol, nicotinamide, nicotinic acid, phenol, piperidine
Uncertain:	caffeine
Yet to test:	~1000 chemicals

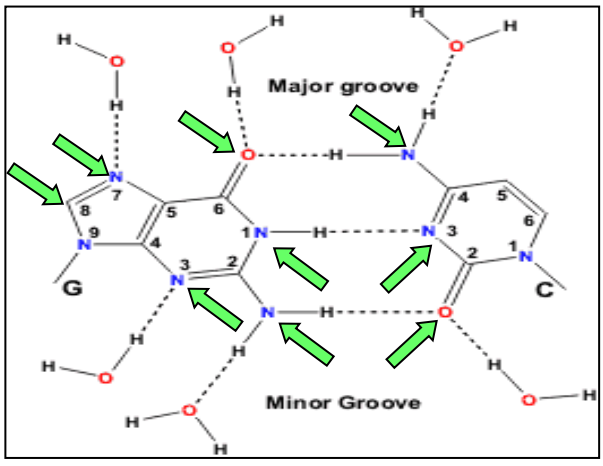
increased levels of cancer. Tobaacco is responsible for 30% of these enviromentally induced cancers. Infection as well if you have repeat infection then you actual get a lot of thing like reactive oxygen species being released in that area and you get different genes unregulated. So infection and persistent infectons can cause cancers as well. There is a table below wher you can also see the less prevalent cases of cancer below. so mainly our diet but there are also other things as well.

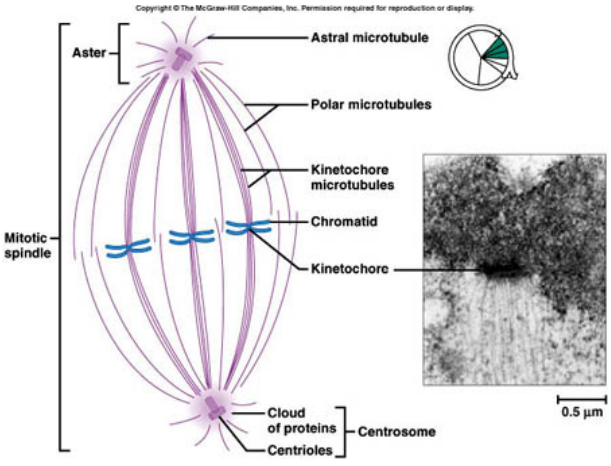
Diet	**35% (10-70%)**
Tobacco	**30% (25-40%)**
Infection	**10% (?)**
Alcohol	**3% (2-4%)**
Reproductive and sexual behavior	**7% (1-13%)**
Occupation	**4% (2-8%)**
Pollution	**2% (<1-5%)**
Geophysical factors	**3% (2-4%)**
Medicines and medical procedures	**1% (0.5-3%)**
Industrial Products	**1% (<1-2%)**

Looking at the bottom you can see that medicines and medical procedures is responsible for around 1%.

So the mechanisms of action and there relation to cancer. So we are looking at chemicals that cause genetic damage and the relationship with that genetic damage with the cancer. This involves three classes known as the

1. aneugens = cause chages in chromasome numeber, either gains or losses.
2. Clastogens = Some kind of chromosome brake
3. Mutagens = Some kind mutation





You can get different classes of chemicals as well that directly interact with your DNA. All the interaction with things like the cellular machinery with DNA replication machinery. So things like Benzo-A-pyrene and the restive oxygen species can come in and attache themselves to the DNA. The other ones like the anuegens come in and attach to things like the mitotic spindles here. So if you get a chemical that comes in here messes up the mitotic spinal, messes up the other one meaning you are going to loose that chromosome. If you mess up both sides then both sister chromatids will go to one side. So you can see how the chemicals can come in and cause these genetic effects. So these anuegens are the reason for the induction numerical chromosomes changes, we are going to learn soothing a bit confusing as well now. So lots of these chemicals here we are going to look at how they give you cancer but lots of these chemicals are linked to chemotherapy as well so its all about concentrations. So the chemicals that we will look at have low concentrations that don't do anything, high concentrations kill off all the cells, concentrations in the middle can cause cancer. So its all about concentration. So the induction of neumerical chromosome changes. So these anuegens are things that affect the cell division and the cell cycle apparatus. To get these gains or losses of genetic material, this is a hallmark of cancer where you could get the gain of a whole extra chromosome and if there is something like an oncogene on that chromosome then it means that oncogene is going to be overly expressed on that chromosome because you will have three copies not two. When it gets hevily expressed you get things like polyploidy, so instead of getting two sets of chromosomes you get three sets of every chromsome. This is going to have the same sort of effect where you are going to get massive up regulation of loads different genes and many of them associated with cancer. So these engines potentially lead to cancer, if your germ cells are exposed to these engines then potentially it could lead to things like down syndrome. So if the sperm or the egg are exposed to things like these anuegens then you get these non-disjunction type of event occurring and potentially that can lead to down syndrome and in the offspring. So the mechanisms tic action of these spinal poisons disrupt the depolymerisation and disrupt the spinal polymerisation or they can mess around with the distribution

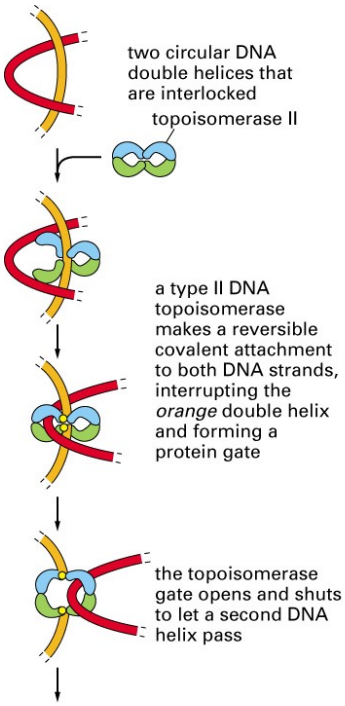
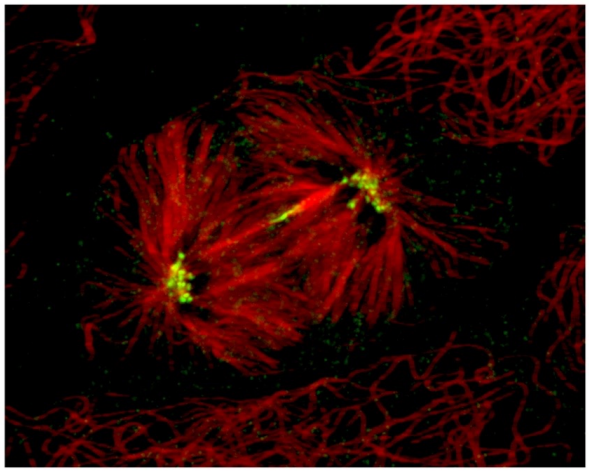
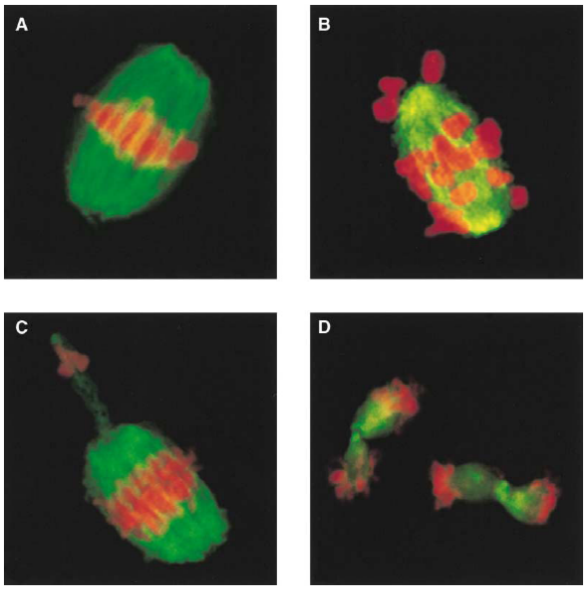


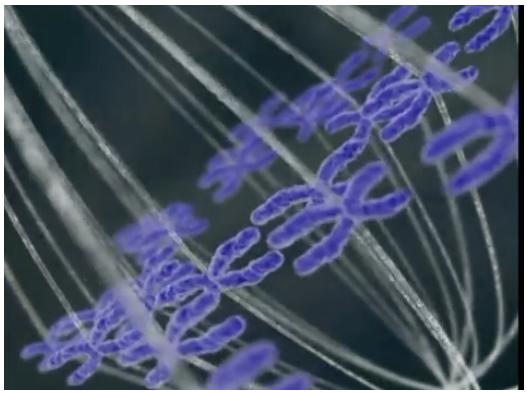
Figure 5–27 part 1 of 2. Molecular Biology of the Cell, 4th Edition.



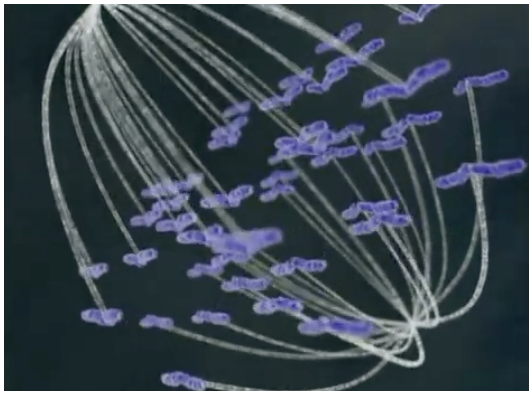


of these spindles as well. So you can get cells to divide into two like this by getting them to pull the spindles to two different sides as seen in the diagram above. You have three or four of these centrosomes that can pull all the DNA into tree cells, four cells instead of two and things like biphenyl A which is present in coca cool can do that. So in the video we are looking at the we are talking about chemicals that can mess up the diffenret processes.

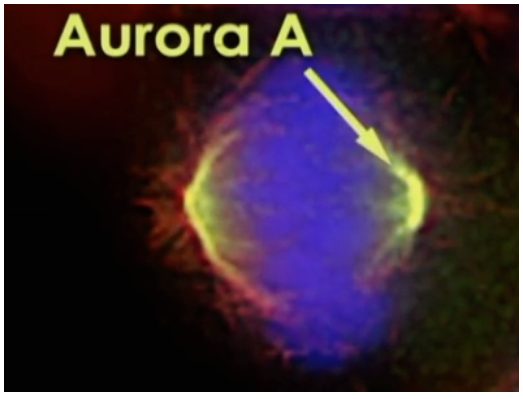
We are looking first at the mitotic spindles coming out from the poles and a chemical can actually stop this polymerisation form happening.



Its a complex process were for every cell division this needs to work properly. The mitotic spindles need to migrate and correctly bind to the sister chromatids at the kinetochores



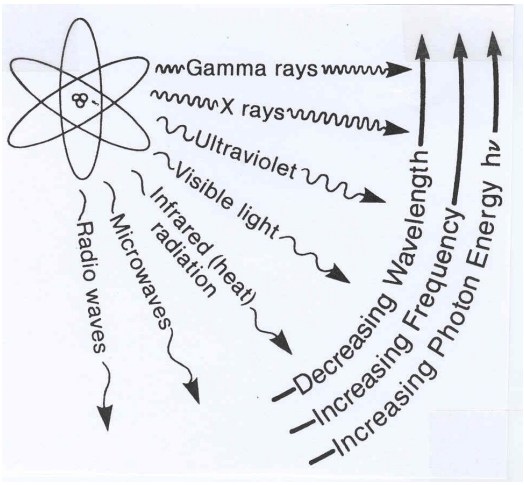
We need to separate the sister chromatids to opposite end of the cell and there are chemicals that can mess these things up.

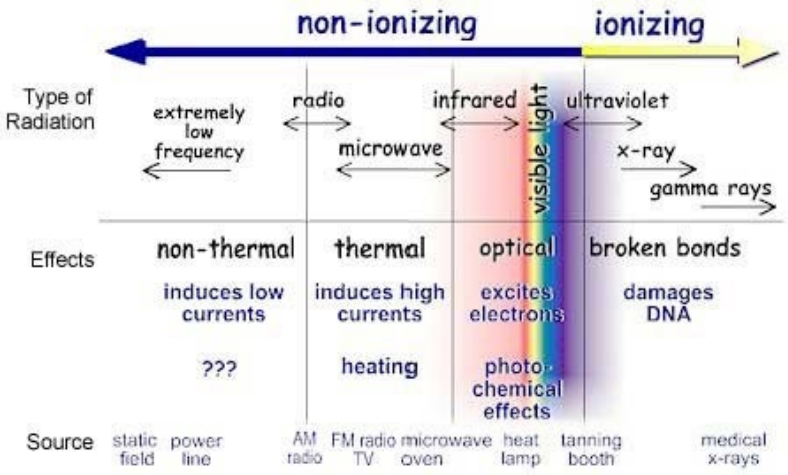


So if you mess up one of these poles then they you can get defects in the Auroras and you can get incorrect division of these chromatids. So just by exposing you chromatids to these anuegens will increase your chances of loosing your chromosomes into the cytoplasm and loosing chromosomes from the cell to your daughter cells. So not really something you want to be exposed too and really

something you don't want your germ cells to be exposed to either because it can cause these cells that are reminiscent of down syndrome and things like that. The kind of places that these things appear. You have a medicine Colchicine used to treat Rheumatic diseases and especially gout which is a highly deadly anuegen. There are some things in bread that can get metabolised in to these engines that are quite happily ingested but again concentration is everthing. So the concentrations we are ingesting makes it ok. Bisphenol-A which has just been has just been reviewed by the europeans increased anueagency has just done the risk assessment on bisphenol-A and said that the levels we are actually exposed to is safe. So it was banned in baby bottles a few years ago. This is because thereby bottles were being but into the microwave and then all the bisphenol-A form the plastic would lack into the babies milk and then the babies were ingest loads of it. So shown that we are normally exposed to quite low concentrations of it. So its all around the place but in low concentrations is the message really. When your assessing this you can see that bisphenol-A dose do these things and it messes up the chromasomes and you get chromosome loss here. It mimics the hormone estradiol in the body causing things like disruption in the hormone pathways. It induces things like chromasome lagging and the chromosome saint attached to the mitotic spindles. Because they mess aroundd with the cell systole so much and they mess around with cell division so much they are quite good chemotherapy drugs at high leves. So at higher levels the cells are trying to divid into two and at a higher level then the microtubules are actually damaged the cels cant divide into tow. So if the cel cant divide into two then your not going to get a big ball of cells forming a cancer because they can't divide. Also if they are trying to divide and they cant a lot of cells just kill themselves so lots of these problems occur then this si a good thing. This is also a bad thing because there not cancer specific. So they kill all your dividing cells in your body which is why you get things like hair loss and internal bleeding from your veins being less stable. The plus side though is that it dose kill the dividing cells in you tumours. So what it doe is induce mitotic arrest in the cells are just stuck in mitosis and this is when you want apoptosis and cell death to occur so you want the tumour to die due to this inability to divide. apoptosis in interphase can also occur as well. So although these anti mitotic processes are becoming affected in cancer chemotherapy they use this limit on molecular effects on tumours of the development of resistance. So sometimes there resistant and the middl of the tumor isn't being exposed to these chemicals and people don't like the dividing effects of all the other dividing cells dining off as well. So there a bit too much of a blanket approach but if you use them in some sort of targeted therapy where people in nano-medicine will say that you can targets drug using some kind of magnets or immunoglobulin ways to target a molecule to your tumor. It brings in high levels of a nasty drug like this so you don't get all of your other cels in your body exposed to this and just the specific tumor ones. So these drugs are used for loads things like the beta alkaloids and the taxes, things form plants so there natural.

So clastogens ar ethe ones that cause chromosome brakes and again a los of genetic material through this chromosome brake is best characterised by ionising radiation such as gamma, X-rays and certain types of UV. Theres some good data on this from the human population so we have one of the best examples is when they first found out the radiation is causing cancer. So in the old days with the old fashioned watches they used aradioactive pants so that your watch is flouresent in the dark and normally the ladies that were painting this on to make a nice clear paint brush when your painting its quite nice to



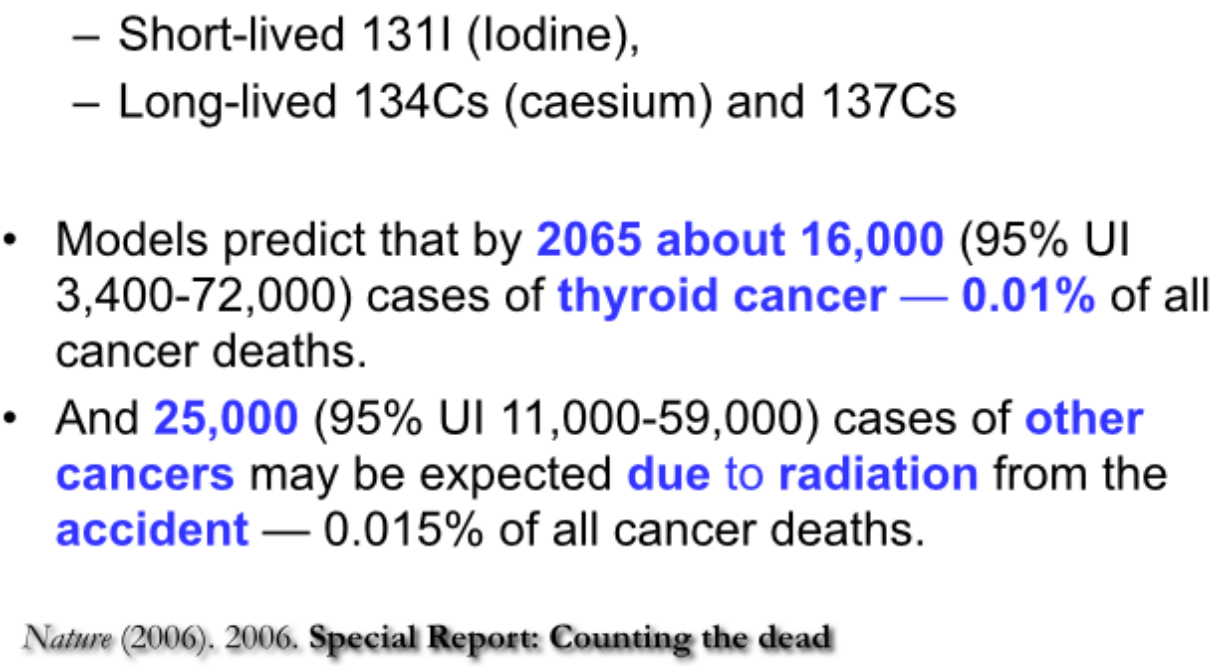


lick

the end because it means tat you can do some nice fine painting. They were licking a radioactive paintbrush and getting levels of young cancers. So it was really linking the radioactive paints to these young cancers. So types of levels that we a are exposed to mainly form radon and if you live in the west country where there are high levels of granite then your going to be exposed to higher levels of radon and when your exposed to these sorts of levels of radon and these sorts of levels of cosmic rays then the people who work in the medical field are exposed to higher levels of X-rays and also in radiotherapy as well. So we are exposed to low levels of radiation but we ar not all getting cankerworm radioation. Some people who were getting cancer from radiation in this example. You cant really do human experiments where you expose people to radiation and see hat happens so the closest control experiments that we have on human experiments are things like the nuclear reactor disasters like chernoble. So what happenedd in 1986 was chernoble had an unauthorised experiment where they were playing around with the reactor. It got too out of control and it exploded and released lots of this radioactive material onto the surrounding population. So around chernobyle there is quite a strong south westerly wind so the cities of Ukrainian-Belarusian were exposed and then a few days later it went easterly and came over towards the UK as well but that was after a couple of days when there were lower levels. So these people were exposed to quite high levels f radiation compared tot he background levels. Particularly the liquidators who were the people who went in to cleaned up the mess strait away. They got exposed to the highest levels of radiation. Then the Evacuees were the people who had to escape somewhere safer. The next ones down were the residents of the surrounding countries. So people were exposed to quite high levels of radiation so everyone thought these people were going to get cancer and there offspring would get cancer and have congenital abnormalities. The main issue really was thyroid cancer. This thyroid cancers affected mainly children in adolescents because the radioactivee iodien is released here an the radioactive iodine released form the reactor was eaten by cows,

Population (years exposed)	number	Average total in 20yrs (mSv)[1]
Liquidators (1986–1987) (high exposed)	240 000	>100
Evacuees (1986)	116 000	>33
Residents SCZs (>555 kBq/m2)(1986–2005)	270 000	>50
Residents low contam. (37 kBq/m2) (1986–2005)	5 000 000	10–20
Natural background	2.4 mSv/year (typical range1-10, max >20)	48
Approximate typical doses from medical x-ray exposures per procedure:		
whole body CT scan	12 mSv	
mammogram	0.13 mSv	
chest x-ray	0.08 mSv	
[1] These doses are additional to those from natural background radiation.		

concentrated in the milk and drunk by the childern and because they were in these deprived areas they already had iodine deficiencies. So soaking up assive amoutns of iodine all of the radioactive iodine into there thyroid glands, this cauesed people to have mutations that lead to increased thyroid cancer sin these individuals. Really when the WHO looked at this they though there weren't as higher levels of cancer as they thought and there were not nearlyy as may as predicted levels of congenital abnormalities as you would have thought in the offspring of people who were exposed. Really they are convincingly high number in the general opinion where there're a lot of increased levels of cancer. So tho model to predict that by 2065 about 16,000 people will have thered cancer as a result of this disaster and because there is only about a 0.01% of all cancer deaths this is deemed to be quite a low number. The 25% of other cases that are exprcted to be due to radians form this accident but because people say this si a low number then its not that significant. Really people do have a case here because there were so mona people exposed to the radiation and you would expect much higher levels of cnacer. But the WHO says that its not as bad as they previsously thought, and some people say its a good thing because there are loads of animals in this area now and they survive just fine. So there ar loads of molecualr machinsms of these clastogens there breaks and rearrangements cause these genetic instabilities and they can lead to increased mutations. So the thing about clastogens is that when something is classed as a clastogen its all a mutagen as well. So normally its a closetgen and the substance is so reactive it reacts with the DNA causes some kind of strand brake. When something reacts with DA is also likely to cause mutations as well. So a lot of the clastogens are also mutagens. We are exposed to loads of these things as well, so occupationlly chemotherapy nurses and doctors are exposed to these things. Obviously the chemotherapy patients will be as well. When we rethinking about why people get cancer and how they get exposed to these carcinogens, there a risk benefit calculation where is fsomeone is going o die within one year of cancer if they take this drug then tell die in ten years from taking the drug=. Then the risk



benifit of them taking the drug outweighs the risk. So there is a risk being that giving them the drug outweighs the risk. So there is a risk benefit thing that comes into play when you talk about a drug and particularly for one of these terminal illnesses. These sors of risk benefits don't come into play when talking about agrochemicals like pesticides or insecticides because so one wants to die in ten years time because they have eaten the cabbage dcovered in these things. So there is a risk benefit factor that only really applies to medicines. We are exposed to these different things, these mutagens, occupational through people smoking. Again environmental exposure and again food hoping up all over the place. Our bodies actually induce quite high levels of these things as well and one of the collegees in amberca where if you do the risk assessment of breathing the environmental protection agency would actually ban breathing because ther are high levels of chemicals that you shouldn't be exposed to. So they can get a bit over the top with these calcualtions. Thee incetacides we do get exposed to. So the kind of chemiccals that cause these mutations are something becoming and usually drop a bit of themslevs onto the DNA and this case its an ethylatignagent which drops an ethyl group not the DNA. Upon the DNa replication this si mis recognised and the chemical that causes this DNA adduct to occur is misrecognisd so upon the relication you get a strait base pair change. So some other things that cause this are things like cisplatin so again the chemotherapy drug regularly induces mutations, is believed to cure cancer by binding to the DNA and interfering with its DNA repair or its repair mechanisms. It binds to two places in your DNA. Cisplatin binds to two places in your DNA and causes mutations as well. So when we are thinking about genetic toxicology we retaking about chemicals that cause gene mutation and chromosome brakes and chromosome loss. You need to be aware that there are some others as well like non-genotoxic carinogens that don't cause these gene mutations but things like these anti-estrogen ones. So they don't cause DNA damage but they can mess around with your hormone pathways that can cause uncontrolled cell death as well but again risk benefit means that this can be a very helpful drug treating many different types of cancers.

So there is a bit of a paradox with these hormone drugs like tamoxifen. So it is actually geneotoxic and it is a carcinogen but it is not a genitor carcinogen because the DNA adducts that it uses don't cause the cancer. So this si a bit confusing that it flags up that it docent react with DNA and it is also a carcinogen then you would immediately like those things together but in this case where this is not actually the case by causing cancer through other mechanisms. So its a bit of a weird one and leads to arguments.

So those are things that react with the DNA and potentially react with the DNA or the cell cycle machinery. Another type of chemicals that are important to look at not so much in the genetic toxicology pathway but int he reproductive science pathway will be teratogens so usually these thing mess up some sort of developing pathway in the developing foetus. They would so much mess with the DNA but rather something at the protein level and so teratogens like Pholidomide where the pregnant mother will take these substances and it will cause some amlformatiosn in the development of the foetus. \so thalidomide was the best example of this where it was given to pregnant mothers as a morning sickness drug and then they realised the offspinfg of these people who had high levels of this treatment actually had this stunted limb growth. So limb bud development was affected. So what it dose is it docent react with the DNA it reacts with cerebrlon important for this limb formation (it actually reacts with the Shh pathway and the reduction in cholesterol). So there are loads of other ones of these as well so when your pregnant and someone says don't take this it is mainly because it can act asa teratogens. So the same is true for all of these things in the table below:

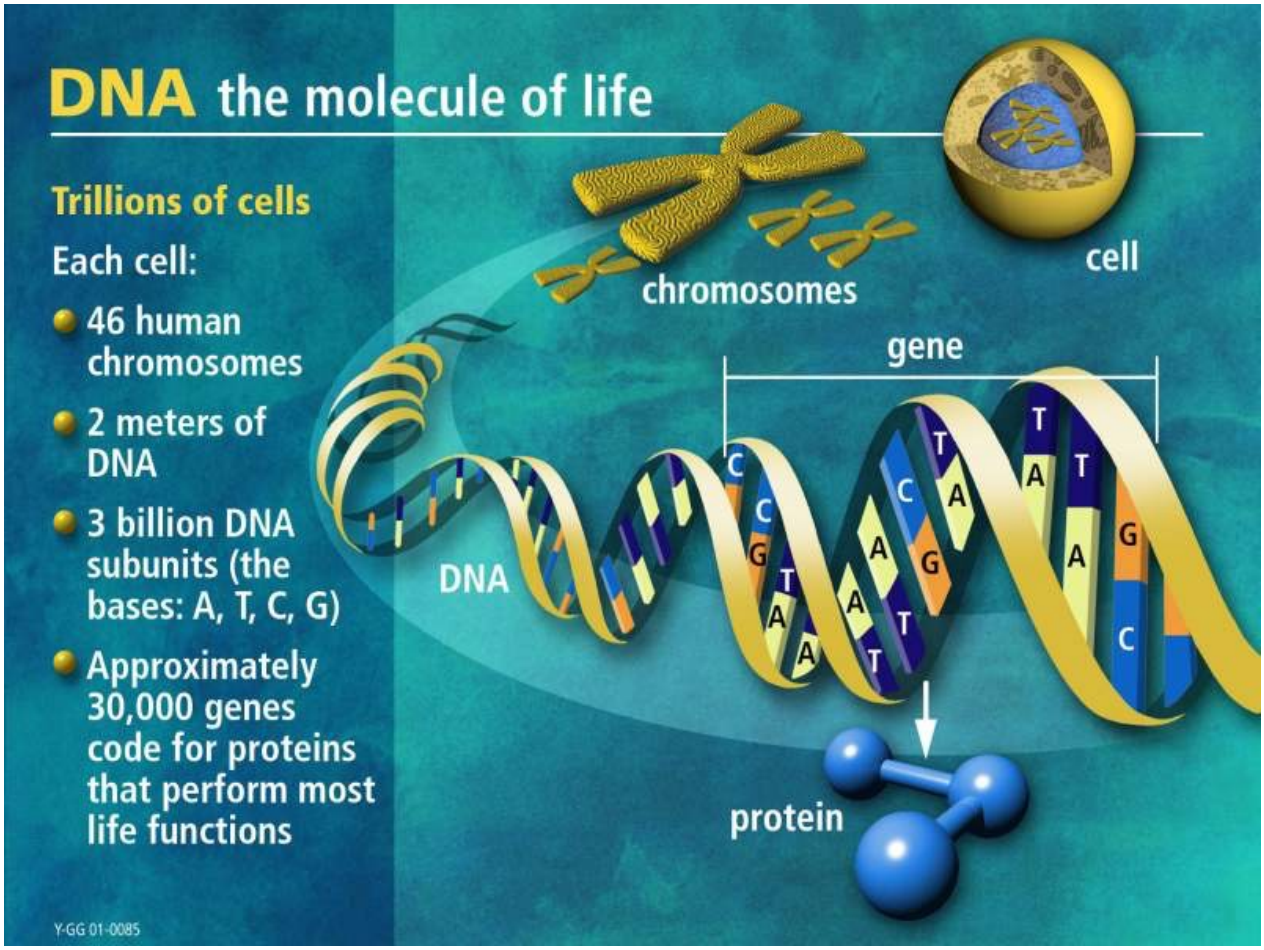
Type	Class	Agent	Risks to Embryo or Fetus
Chemicals			
Pollutants	Heavy metals	Methylmercury	Teratogen
Recreational drugs		Ethanol (alcohol)	Teratogen, growth retardation
		Nicotine (tobacco)	Death, growth retardation
		"Crack" cocaine	Death, growth retardation
Over-the-counter drugs	Salicylate	Aspirin	Death, growth retardation
Drugs			
Anticancer agents	Folic acid antimetabolites	Methotrexate, Aminopterin	Teratogen
	Alkylating agents	Cyclophosphamide, Busulfan	Teratogen
Antibiotics	Tetracyclines	Tetracycline	Hypoplasia and staining of enamel
	Aminoglycosides	Streptomycin, Kanamycin	Hearing defects
Anticonvulsants	Oxazolidinediones	Trimethadione, Paramethadione	Teratogen, death, growth retardation
	HyDNAtoins	DiphenylhyDNAtoin (phenytoin)	Teratogen
	Carboxylic acids	Valproic acid	Teratogen
	Bromides	Bromides	Growth retardation

Chapter 2 (Genetic in genotoxicology)

This chapter contains

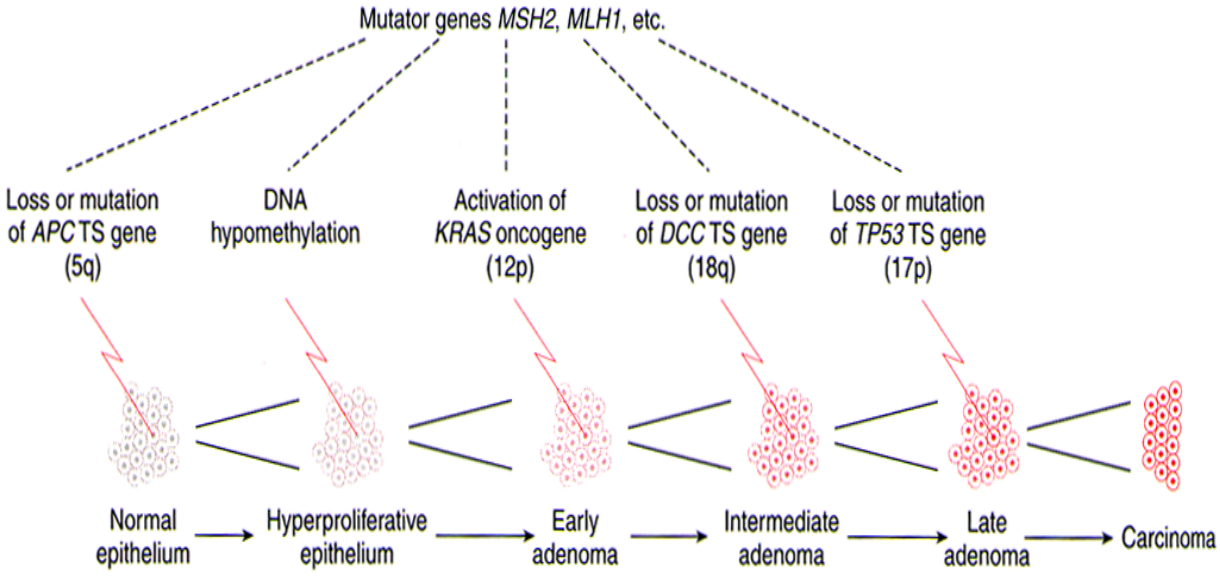
- Introduction to genetic toxicology
- Ames tests

Introduction to genetic toxicology



So we are talking mainly about DNA but not exclusively its a big target and we are looking at test that we do are looking at the changes to DNA and as we say it is a big target and you've got something like three billion sub units of nucleotides in each cell. So there are plenty of things that can go wrong or that could go wrong. So what is genetic toxicology? Well back in the good old days when it first started it was talked about mutagenicicty testing but we are not a=only testing for mutatiosn. We are also testing for changes to DNA as well and changes to the genetic apparatus and not all of those will lead to inherited mutatiosn. So we now tend to use this broader term genetic toxicology or genotoxicity testing which not only include mutagenicicty testing but it also includes chromosome abnormalities that could be structural or numerical changes. DNA damage affects on DNA repair and damage to the cell division apparatus that can lead to chromosome loss or gain or aueploidy. So why do we do it, well if we look at the pharmaceuticals for example then most pharmaceuticals will be tested at some point in cancer studies or rodent, raddit and mouse cancer studies. Only drugs that ar used for diganostic porcesses or very short

term use will not need carcinogenicity testing because those tests are time consuming and expensive. Pharmaceutical manufactures want to get some ideas of wether the drug is going to be effective and they want an idea in clinical trials firstly in healthy volunteras and then in patients to see if the drug works. So we need some safety data before we go into human trials or human patients, it would make the drug development process ridiculously long if we had to wait for carcinogenicity data before we could do those clinical trials. So we used geneotxoicity data as a surogate and it is an alternative and gives us a good indication of cancer potensial before going into hese types of clinical trials. For industrial chemicals like household products, often they are made in such small quantities that they dont require carciogenicity testing so we dont use geneotoxicity data as an indicator of cancer risk without having to do the cancer studies. In cosmetics we cannot do any in vivo testing so we cant do cancer studies, so we have to relyy on cancer genotoxicity data to tell us about the safety of cosmetic readings. Agrochemicals ina way similar to pharmaceuticals, most of them will eventually be tested in cancer studies but again the companies developing these want to find something out about how affective they are. They want to be able to do field trials and we use genotoxicity data as a predictor of safety in terms of cancer risk in order to get those trials underway. So the genoxoicity testing is not only about predicting cancer. The examples just mentioned are mainly too produce cancer potential but DNA damage and mutation have other health effects. So although mutation is most often involved in the initiation of tumours. IT is also involved in tumor porgressio so there is a loss or gian of tumor material. There are other conditions such as arteriosclerosis and inobrn errors of metabolism that also originate from mutations. In terms of chromosome damage a lot of spontaneous portions and birth defects are associated with chromosomal



dmaage. So if we look at this below it is a cartoon of colon damage or colon carcinomas. Some cartoons have 5,6,7 steps but this si a 6 step process. Basically what we want to focus on here is there are mutation, DNA modifications this is now in the field of epigenetic. There are alterations of methylation patterns. Activation of oncogenes and then loss or gain of mutation material hat might be through

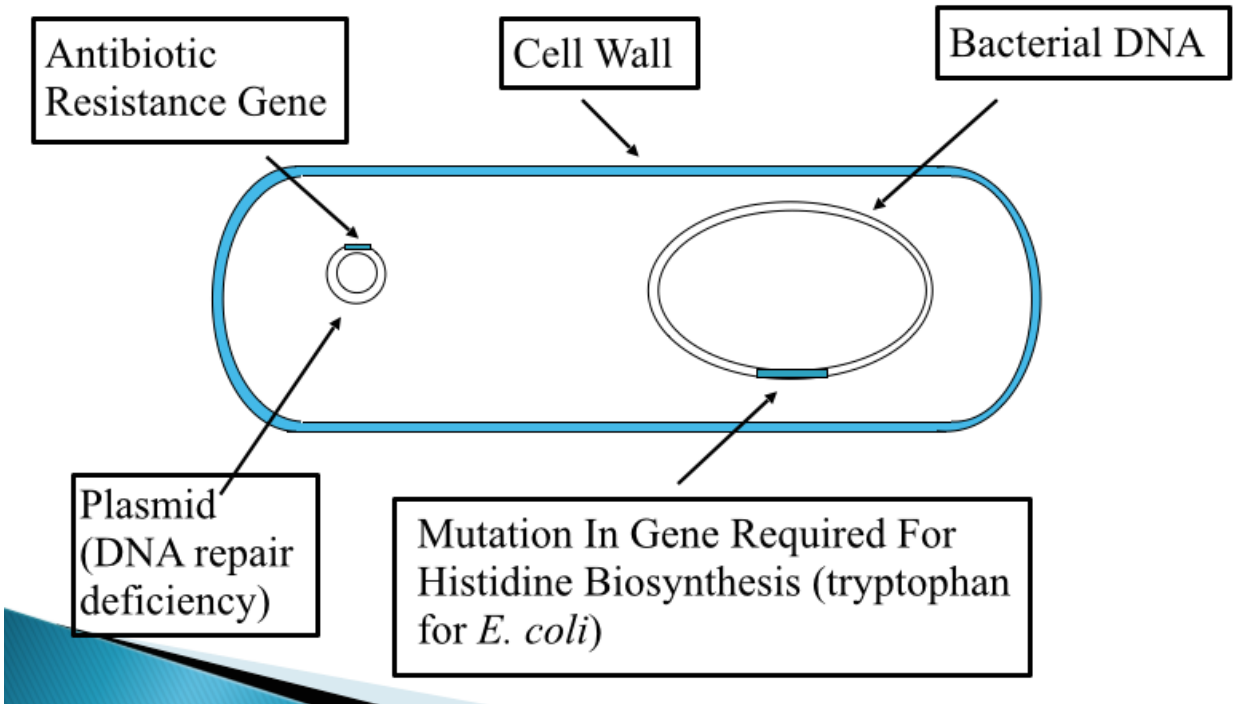
chromosome deletions or rearrangements or even chromasome loss. So there are mutation involved not just in cancer but in a whole host of other health defects and again as well. In terms of cancer causing genes there are four main types of genes that are involved somewhere in cell division and most tumours have a faulty copy of one or more of these. So oncogenes , tumor suppressor genes, suicide genes and DNA repair genes, the fact that we find mutation in these genes in cancers and tumours is reanforcing the idea that mutations are invovled in cancer. So because there are several different types of even involved in cancer and in other genetically derived health problems we need to be able to detect a number of diffenret health changes in any gene toxicity test that we do. We need to be ablate detect the mutatiaons structure of the chromasome damage and numerical chromosome damage. But there is no simple test that can detect all of those. So we have to use a battery of test that compliment each other. This means using either two or three in vitor test. This si usually the first step and the cometic will be the only step for hazard identification. For many years we have actualy used three different test the latest recommendations now form the UK and the european safety authority of minimum factoring that covers all of these end points is an aims test and an in vitro micronucleuse. The ames test picks up mutation and the in vitro micornucelase picks up both structural and neuterical chromosome damage. Now because these are in vitro hazard identification test we really push the limits on these test where we test with very high concnetratiosn and some very extream conditions. We also add a rat liver extract called S9 to mimic the metabolism of mammalian livers. The bacteria and cells that we use in culture do not have much in the way of metabolicc capablety so we need to simulate what will and can happen in the whole human or the whole human. So we have this metabolising mixture. All of these features the high concnetrations or the extreme conditions and unusual metabolsing mixtures can lead to artifacts. We need to do this to make sure we are not missing anything important, we are not failing to predict the hazardous chemical but there is a potential for artifacts when we go into these unusual conditions. In terms of david kurklands work as a consultant is to try to work out wether the results that are being seen are a true reflection of a hazard or are due to the extreme conditions that they are using. We are creating physiological stress abnormal conditions in that they are not necessarily of any hazard. So teasing out the real negative from the valse negative and the treu positive from the false positive is still a vary freequent even and maybe 1/3rd of all the test that are done will produce an unexpected or an inconsistent pattern of results that needs to be evaluated in some way to find out wether we really are looking at a hazard or wether we are lookng at some sort of smoke screen and we are having to deal with it. Once we get past the in vitro test for everything apart from cometic ingredients we usually do some follow up in the in vivo tests. Now these are usually hazard characterisations. The first thing we are asking is what ever we saw in the in vitro test, is it reproduced or dose it also occur int he whole animal. So for example if we have a positive result in vitro do we see that positive result also in vivo. If the chemical we tested in vitro was negative then by doing an in vivo test we get the opportunity to see if there are any unique in vivo effects. There might be for example a kidney specific metabolite produced and we haven't used kidney S9 in the in vitro tests. There could be reductive metabolism by the gut bacteriam which we have not covered in our in vitro tests. So the in vivo gives us the example to see if there ar any unique in vivo tests even if the in vitro tests were negative. We now have quite a wide selection of in vivo test that we can do. Covering the same sort of end points that we could test in vitro. So we can look for the mutations in target genes usually in transgenic animals and we can look at chromeasomal damage and numerical mage by

minornuclei and we can do a couple of other things by looking for effects on DNA repair. We can look for effects on DNA strand breakage and we can look for the formation of DNA adducts and we will cover the formation of all of these in these couple of lectues. So in all of these in this first talk we are going to focus on new new patient data and we ae going to be talking about three different types of test. Here in the mutation in mamalian cells and mutation in animal models. The in vitro test with he bacterial and the mammalian test are widey used and the bacterial testing in particular. The transgenic mutation test are less widely used because there very expensive. Orangenic animals and transgenic mice cost something like £200 each per animal so if your doing the transgenic mutation test your looking at probably getting on for £100,000 and there fore companies do not underake those kinds of test unless they have too. So we are going to pause at this point before we describe the test. If heading in to industry then these are the sorts of test that will be carryied out and and that it to try adn establish wether a compound is are for human exposure without having any cancer data for example. Then you need to be able to rely on the results. In order to do that we have guidelines which are accepted worldwide to all countries that sign up to OECD (The Organisation for Economic Co-operation and Development (OECD)) There ar about 60-70 countries that are part of the OECD and this si to maintain consistency. So that if you test a compound in a lab in china using an OECD guidline then you test that compound in south america then it should give you the same result because you following a recipeie. ANd trying down the recipe for giving those reliable assaults is what these international guidines are about and its what indusry has to do in order to produce results that are going to be produced to the regulating authorities. Wether its the health authorities or the food safety authority, They can rely on it if you follow those protocols. So over the years those protocols have become developed and have in many cases become slightly more intensive and slightly more benanding than they were ewehn they were first introduced back in the 80s. So what we are describing is what we now believe ar the best ways to do those tests in order to produce reliable results. The next one is industry contract labs and so on and what they are doing day in and day out in order to report the faciliaties as being accepted as reliable. Now having said reliable we are pushing the boundaries on these tests and there is the possibility for artefacts.

Ames tests

so this is commonly referred to as a rever mutation test.So what are we doing is by starting with bacteria that already have a mutation. Its down in one of the pathways associated with amino acid synthesis and in the case of the salmonella bacteria its used with histamine biosynthesis and in the case of E. coli its tryptophan what we are looking for is wether by exposure to our test chemical wether we can revert these bacteria form auxotrophie to prototrophie so that they can fully synthesis the amino acid and they dont need the suplement. So we start of having to grow the bacteria in histamine or tryptophan containing medium and then we look for the phenotypic change to the ability to grow in the absence of the additional amino acids. There are several different strains of bacteria that we use. Each of them we use that has a very similar and small genetic target and because the targets are similar in those strains we need to use several bacterial strains in order to cover th edifernet specificities in order to cover the mutagens that we are looking to detect. In addition to this small specific selective target the bacteria

have been engineered to be more sensitive than a normal bacterium would be by the addition of several other traits. So they can contain DNA repair deficiencies so you can cause damage to DNA and its not repaired its not recognised therefore its more likely to end up as a mutation. The cell wall of the bacteria has been modified increase its permiability to large in particual chemicals. So they can actually gt into the bacterial cell and actually interact with the DNA. A number of the strains that we use also have plasmids that confer and increased susptability ot mutation with out a concomitant sensitivity to toxicity. So this helps us separate the toxic form mutagenic effects in terms of dose range or concentration range of the chemical thats being tested. So just a cartoon of the bacterium seen below.

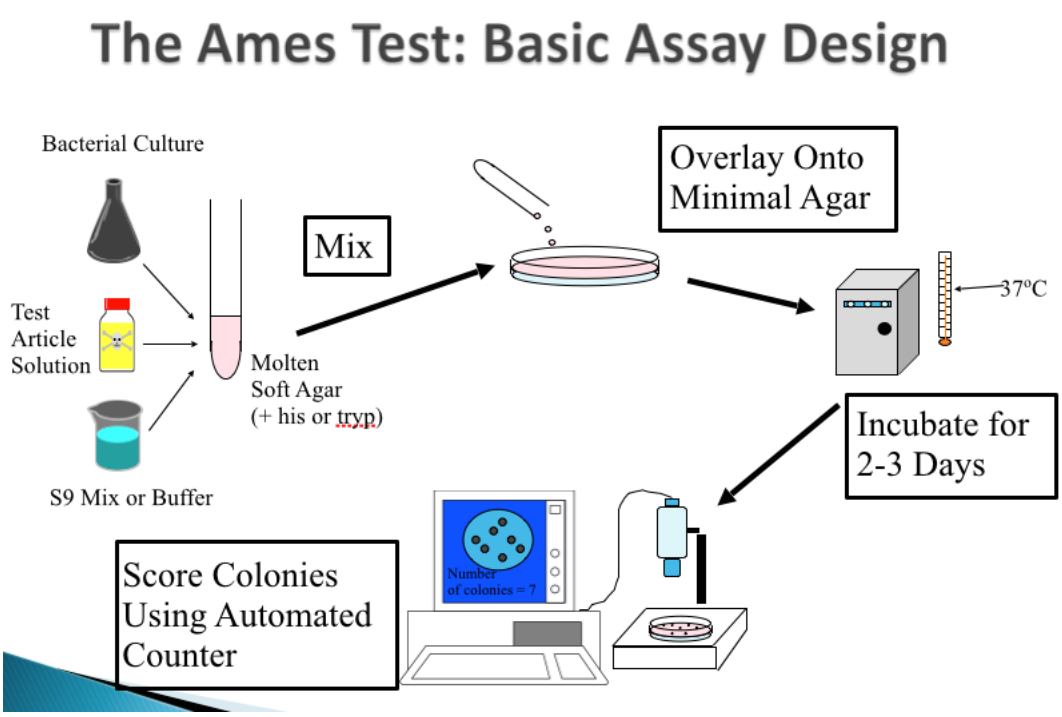


Theres a mutation somewhere in the histamine or tryptophan biosynthesis pathway. The cel wall has been modified to allow large molecules to enter, some of thes bacteria have this plasmid with the DNA repair mechanism deficeincy incorporated on to it. This biosynthesis gene is simply a marker that allows us to check that the bacteria still has the plasmid if they are resistant to the antibiotic then we know the bacteria has the plasmid. Another aspect of these bacterial tests is that the site at which the mutation has been implemented which in the case of salmonelle is in the HisG HisC or HisD genes. These are hotspot for particular classes of mutagens, in the case of these three markers for GC base base pair or mutations or alterations of the GC base pair. We are able to check what has happened to the DNA sequence around the original mutation by sequencing the DNA in that region and these bottom four shown in the table below here (the HisG46, the HisD6610, the HisD3052 and the HisC3076) are all strains with GC target mutation so these strains will pick up things like base pair substitutions so you have the TC, frameshift mutations with the addition of a couple of cytosines here. Frameshift here with the deletion of a cytosine. TA1537 also picks up a formativet but its more but he way of intercalation. The G428 strain has an AT target so your looking at open mutation and theres quite a wide target and range of changes

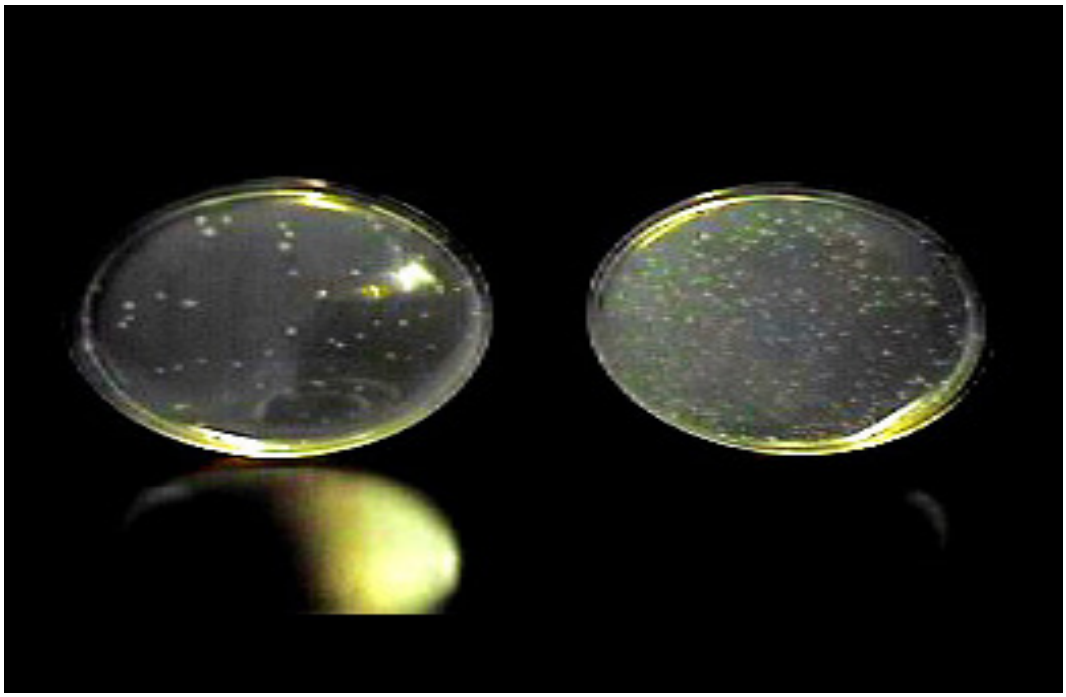
Mutation	Strain	Nature of mutation	Reversion events
hisG428	TA102 TA104	CAA TAA (ochre) (*E. coli* WP2 also detects ochre mutations)	Transitions, transversions Extragenic suppressors Small deletions
hisG46	TA100 TA1535	CTC CCC	Base-pair substitutions Extragenic suppressors
hisD6610	TA97	ACC-CCT (opal) ACA-CCC-CCC-TGA	Frameshifts
hisD3052	TA98 TA1538	GCC-CGG GCC-GGC (opal)	Frameshifts
hisC3076	TA1537	Not known – presumed +1 near CCC	Frameshifts

that occur in these. The TA102 or TA104 with transitions or transvertison ETC. So although these targets are quite specific and we know what we expect to be going on when we actually look at the range of changes that occurs in human bacteria. So for example if we take TA102 the HisG428 then we actually find that tater ar multiple changes that have occurred in order to achieve this reverse mutation. so we see a true reversion which is a simple base change from aT to GC. But we also see the inorganic suppressors have been mutated or there have been large deletions with 3-6 base pari deletions and we can also get mutations in extragenic supressors. So although these are supposed to be quite engineeried to be very specific and very selective mutation that would probably only be reverted by. One single event we actually see multiple events particularly int eh HisG428 strains with these ochree mutation. We mentioned metabolic invasion and of course bacteria don't have livers. In humans your exposed to a strange chemial and the liver sees it as not being particularly useful and tries to preparee it so that it can be excreated and that means that in the liver these compounds are metabolised and particularly in the liver it tries to make them water soluable and if you have got an organic chemical and you have ingested an organic chemical, your liver want to make it water soluble so that it can be excreted in the urine to get rid off it. Now that metabolism is generally oxidation metabolism. sometimes it makes a mistake so if you breath in bozo-a-pyrene or if you swallow bozo-a-pyrene for example your having a barbecue, having a bonfire, eating the same time. You take in some bozo-a-pyrene and your body docent like it and tries to get rid of it so it metabolises it and wants to make it water solublee by adding hydroxy groups. In the process it makes an epoxide and that epoxyde binds to DNA and that is what is mutagenic and carcinogenic from bozo-a-pyrene. So in the process of our own livers trying to detectivey our bodies form that chemical it actually makes an intermediate that binds to DNA and causes mutations. The body is only trying to do the right thing by removing the chemical but in the process of doing s there is a DNA reactive intermediate or a idol-epoxide which binds to DNA and causes the mutations. So it is important that we include some aspect of metabolism in to the testing. So we take some rats and pretreat them

with this either the polychlorinated biphenyl or a mixture of phenobarbital. These substances stimulate the liver to increase the enzyme level some of the p450s and in particular p450s involved with oxidative transformation. So the S9 is a 9000 G supernatant from the livers of the rats that have been pre-treated with this compound. This supernate contains mainly high levels of mono-oxgenases, oxidases and aminidases etc. Some of these p450s are amplified 40 times the level that you would find in a normal liver. so they really are hyped up to try to push any metabolism in identifying the hazard. Now the S9 wont do the work on its own, you need an energy source and you need co-factors so we have to add those into he mix as well. So when we are going an experiment we are growing batchria overnight where they are unsually diluted so there inactive growth because our test chemical. We have our S9 and co-factors or we also do the treatment in the absence of the metabolism if there are any direct effects. So for example an alkylating agent like methylmethol sulphanate docent need a metabolism. It will cause mutation without metabolism so we will pick that up better if we pick it up in the absence the S9 mix. So we do it with an without the S9 mix and there are two ways to do the test, we can mix all of these together in a coupe of MLs of our soft agar. Mix it all togather and pour it on a plate and apply the colonies. Or we can mix these together without agar for about a hour half an hour maybe and then add the agar and do the plating. So without the agar its called pre incubation and of course what this dose is it you can have much high concentrations of both test chemical and the liquidd S9 and the bacteria. You also have a completely liquid matrix, so you increase the chances that the compound can get into the bacteria or that a metabolite can ge tinto the bacteria but you also increase the chances of toxic effects. So there are some advantages to doing pre incubation but there are also some downsides to doing pre incubation where it can be very toxic. Now you will see this little bit here in the slide below that in this cocktail we have a small amount of histadine or tryptophan. Can anydy tell me why they think we need to have that? We are starting with bacteria that are deficient in the ability to synthesis histamine or trypotuophan so we need to make sure that those bacteriaia go through a few cell divisions cause any damage to the DNA that our test chemical has caused needs to be fixed. So it needs to be in a permanent state or a reliable state where the changes tot eh DNA need to be consistence and persistent and that change then needs to be decoded intohe various enzymes that are needed for the histamine biosynthesis. So the damage to the DNA needs to be consistent and the RNAs



and proteins that are down stream form that DNA need to have had time to change. So you need to give the bacteria time to got from a mutation that means it cnat produce histamine or tryptophan to a native DNA that can produce histadine and that means you need the bacteria have to go through a few divisions. So in order for them to make that happen we need to include a little bit of histadine or tryptophan in this mixture. Now it is only a little bit and when the bacteria have used it all up which they will quite quickly then only the mutant backteria will grow. The starter bacteria that have not mutated cant grow because they have run out of the little bit of histadie or tryptophan that we put into the cocktail. Thats important as we will see in a couple of minutes. So These bacteria, we normally put about 10^12 bacteria on a plate and because they are engineered to be so sensitive then even on a control plate we get a number of spontaniouse mutation. So this jazzy are in the background are the starter bacteria that cant grow in the present of histidine, they have gone through a few divisions in the presence of histidine



that we have include and then that has caused them to stop growing. So this is a lawn of micro colonies. So they are starter bacteia they have not mutated they have gone through a few division and then they have stopped dividing. Only those that have mutated and are now independent of histidine that can synthesis there own histidine to grow on to form discreet colonies that we can count. so this is a part treated with a positive control chemical and as you can see there are a lot more colonies and a lot more mutants. Sop it just becomes then a numbers game. We need to treat enough cultures we need to treat enough concentrations and we need to treat a number of replicates. We wont dwell on it, this is just a summary for easy reference if you wanted to look at it. Because the different strains have different types of mutation we are going to cover every possible type of chemical damage to DNA. Then we need to use a number of diffenrt strains and usually ther are four strains of slmonella with a GC target so these strains are

- TA1535
- TA1537
- TA98
- TA100

one or more strains that can detect mutations acting at AT sites so this would either be

- TA102

or one of the E.coli strains like

- WP2 uvrA
- WP2 uvrA pKM101

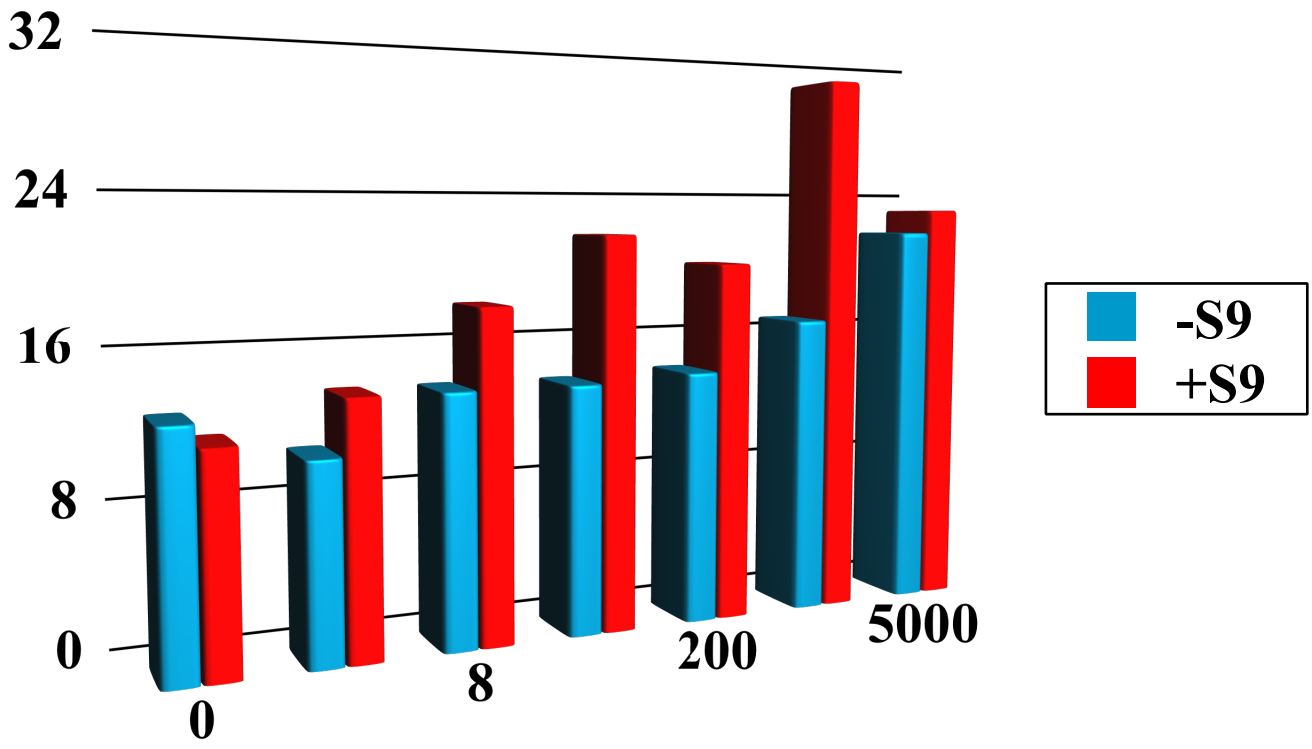
- Need basic 4 strains of *Salmonella typhimurium* (G-C sites)
  - TA1535, TA1537 (or 97 or 97a), TA98, TA100
- Plus 1 or more strains to detect mutagens acting at A-T rich sites
  - Either TA102 or *E. coli* WP2 *uvrA* or *E. coli* WP2 *uvrA* pKM101
- Each strain detects different effect
  - +ve in only 1 strain indicates hazard

And because each strain detects a different effect we only need a positive result in one strain to indicate a hazard. We often getting niece clients saying well we tested it in 5 strains and four of them were negative so that outweighs the positive well no this is not scored one strain thats positive is telling you something important and we are not looking at dull efforts. So its important that plate incorporation and pre-incubation are both available. We mentioned there are pros and cons for each but from an ideal scientific point of view you would actually do a test using both methods but now many of the guiltiness tell us that we only need to choose one of these and that will be ok.

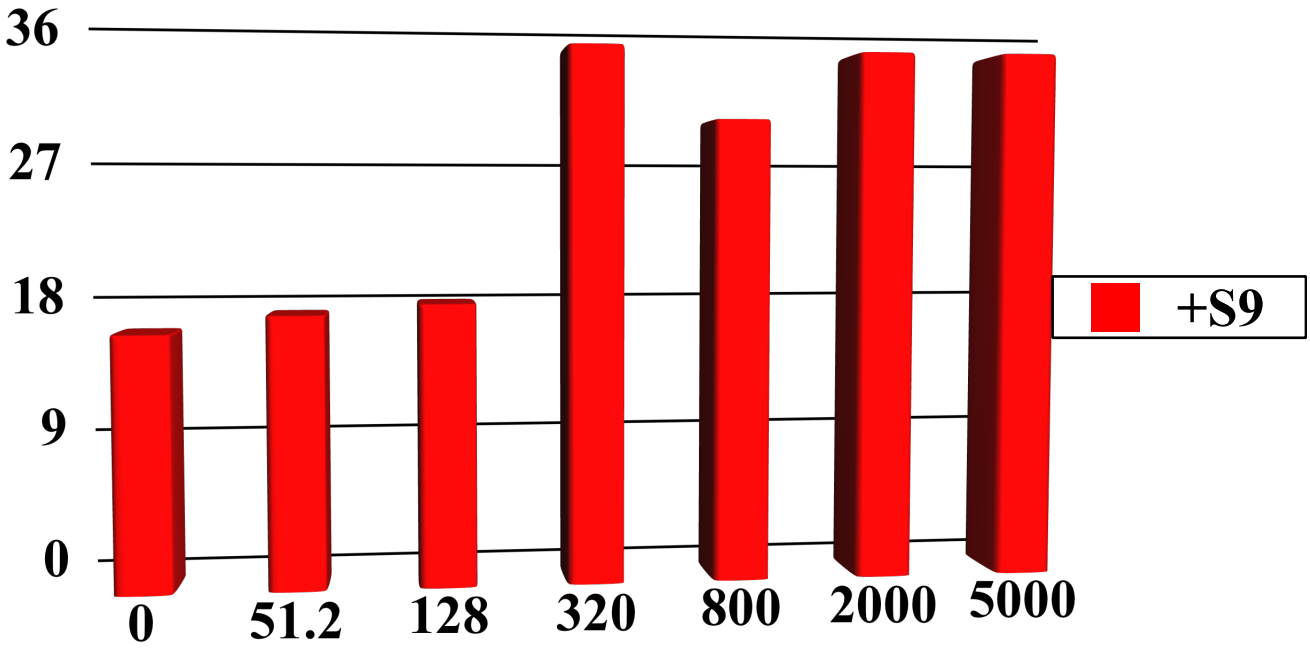
As we mentioned earlier we do go to extreme conditions with testing in 5mg of platelet which is quite a lot of chemical. We can test insoluble concentration, we can test some compound s that precipitate as long as it dosnert interfere with the story and this is quite useful because it can allow us to detect impurities. It is very difficult to synthesis a product and make it 100% pure technical materials are quite common levels of impurities are quite common and they may be mutagenic. It is helpful from a health and safety point of view to know that thats the case. Generally in the ames test we do three repliacates per concentration and five concentrations and we do the test with a n without these metabolising agents. Now as we have said if it was an ideal world then we would suggest that if you do an experiment then you should repeat it because scientific rigour suggests that results need to be confirmed and this would be needed to change the conditions to do both plate incorporation and pre incubation but for human pharmaceticals this si now not needed a single robust experiment that is clearly positive or clearly

negative dose not need to be confirmed. If you do do a second experiment the best thing to do is not an identical repeat but to change the conditions like the pre incubation to plate incorporation or the concentrations of the test chemical or to change the concentration of the S9. As long as you have fourfold all of the parameters that are in the guideline with enough replicates and enough dosing then a negative control and positive control and full accounts of the colonies then should be an acceptable complete experiment. ames test have been around since 1973-4 and is still at heart of all regulatory process. People say bacteria don't get cancer but these are very good indicators of cancer potential.

Wesaid that we need to add a bit of an amino acid to get the bacteria through a few divisions with the mutation.Fix it in the DNA and allow then for the downstream RNA and protein changes to occur. So basically just allow the bacteria to synthesis histidine or tryptophan for themselves. What do you think happen if the substance that we are testing contain one of those amino acids? What if we are testing a plant extract that contains amino acids or the pharmaceutical industry is synthesising a peptide to treat maybe some kind of genetic disorder and that peptide contains some histidine or tryptophan, whats going to happen? It will basically grow on the treated plates where we have got more histidine or tryptophan the bacteria will go through more divisions. Whats that going to lead to? It can then revert back to its original mutant. First of all if you get a lot of growth of bacteria then that background lawn of growth is going to be vast and you wont be able to see the mutant colonies. Before you get to that point, the bacteria on the treated plates so with he amino acids will go through more divisions before thats used up. For each time a bacterium divides, there is a specific chance that it will mutate so the more divisions it goes through the more spontaneous mutations it will occur. So you end up with what looks like a dose responcewhere you end up with increasing numbers of revertant colonies on the plate that is just die to feeding. So the histidine is allowing the bacteria on each of those treated plates to go through more division before its used up and with each division theres a chance of a psontanousoe mutation so its not getting mutation due to interactions of the test chemical with the DNA it is simply feeding the bacterium. which is increasing the frequency of spontaneous mutations and you can fiddle and change the way you do the experiment. you can do a treatment placement experiment. Whereby after treatment it would be the preperation that might ocntiain the amino acid. You can spina and wash and take the test chemical out of the test chemical before you put it on the plate and then it cant feed the bacteria. So you can reduce the risk of basically getting a fals positive because its not a mutagen its a food source. People particularly make products form plant extracts and of course int he chemical industry it males oils and fats and proteins form plants that are supposed to be good fro your skin and your hair and that sort of thing. They can cause problems in this kind of test. So some audience participation. This is a test that was done a few years ago with strain TA1537 that just using plat incorporation methodologies the Red bars is with S9 and the blue bars without S9 where we got up to the extreme of 5000ug. seen below we at that time used to do statistics quite regualrly and this distinction in the ames test can pick up increases of less than two fold that has been statistically significant so this was the first experiment we did and if you were doing this in the lab and you had to go tell your boss down the corridor that the ames test got this result. What would you be telling him? Is it positive or negative not sure? It looks positive because you certainly wouldn't be telling him it looks compliantly negative so we did the right thing by doing the experiment

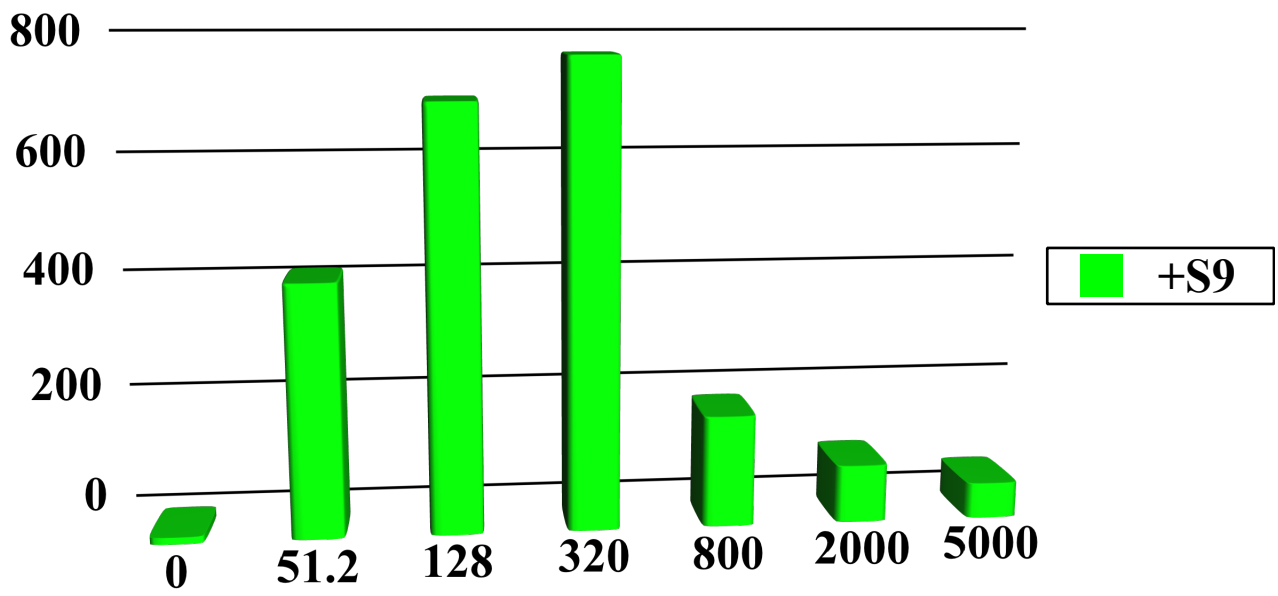


again and we changed the dose range. So we closed in on the area that the dose range that we sa something and it was rproducible and now instead of doing something at one dose point we have closed up the dose range this is the diagram seen below. These are all statistically significant with all about twice the background. So negative positive? it is reproducedle so it looks positive but what we have to think



about is the general rule of thumb for this strainn is that you only accept a threefold increase as being biologically significant. Now that is because the spontanioue counts with this strain can be anywhere between five and fifteen or five and twenty. So you can get a three fold differences just in your control

plants and therefor tend not to accept that a responsee in that strain becomes biological significant unless its gone up more than three foldd. So this reset would not be positive even though its reproducibel and even though its not a clear negative. What would you do with something like this? We have done plate incorporation. This should be made clerer that this strain is specific to thea effects of this compound where all other strains were negative and this one was the only one that saw an increase and it was only present in the S9 positive tests. So we could pal with the S9 concentrations or we could try a different S9 product. We could do pre incubation now then. so thee results are what are called weak positives and it is less than threefold, its not however clearly negative but we wouldn't however get overly excited about it. Now we did a pre incubation experiement and we got this graph below.



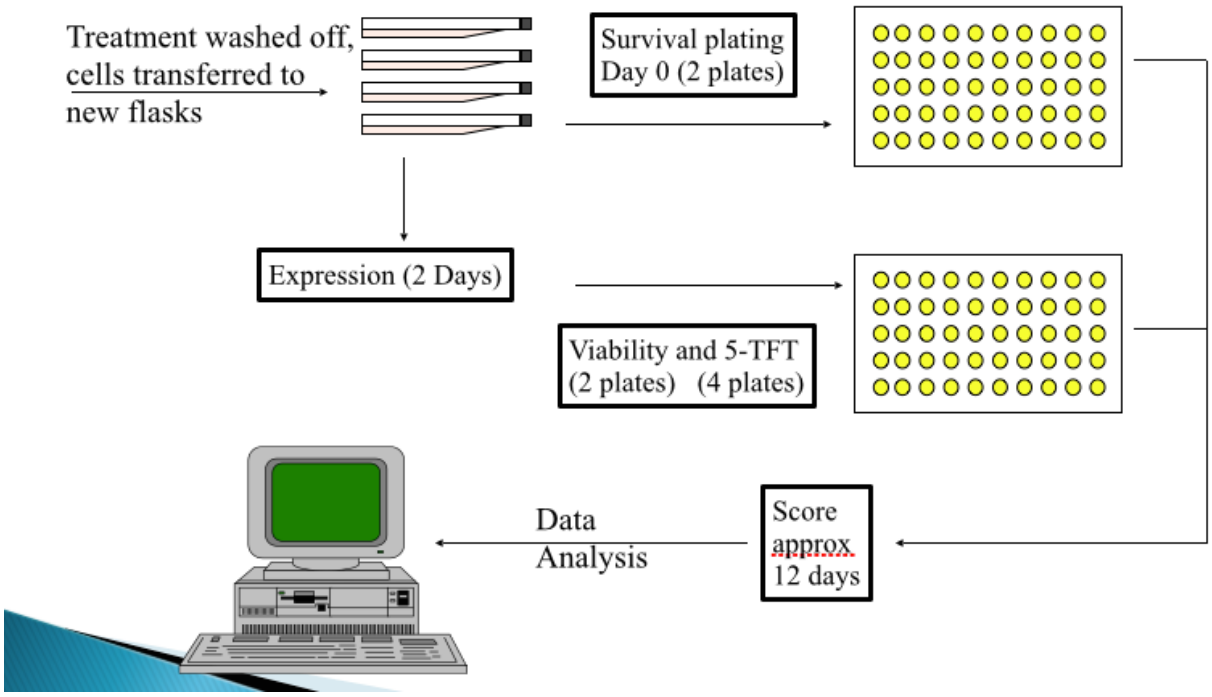
So we are at hundreds and hundreds of mutations and fold increase in the mutations just by doing pre incubation and its because of result like this that we should worry when the regulators say well we can choose pre incubation or plate incorporation it dose matter pick one and stick with it. This ia an example of a stark difference when the difference between the mutagenic activity between the mutagenic acitvity between - and + S9. This then was explainable where this compound was a anthrequinone. Now anthrequinone are flat molecules or palnear molecules and they can easly slide between the base pairs in the DNA and thats what TA1537 detects it detects a frameshift mutation by a chemical slotting in between the base pairs an distorting the helix and thats what this was doing after metabolism. It needed metabolism, it probably needed one or two of the side groups to be knocked off so that it could be slotted into the base pairs. Now unfortunately we are in the contract industry and we often don't get to follow up on these compounds so we don't think this compound was ever progressed and we don't think this compound was ever testeed for carinogneicity. We don't know wether that strong positive ames test was an indicator of cancer potential because it never got that far and thats one of the shames because sometimes it gets some really interesting results and you don't get to follow them through so you don't get any significance of it. But for me that was a stark example of how if we had only down the plate

incorporation test then we would probably have said this is pretty weak, that might be due to an impurity. Lets synthesis another batch and do our in vivo test and mammalian cell and it will probably be negative and it would have got swept under the carpet. And we never knew wether that really strong pre incubation response was a true indicator of a real problem.

So thats all on the ames test and now we are going to do mammalian cell mutation and transgenic mutation. Now we can see things happening in bacteria that may not happen in mammalian cells the organisation of the DNA for example is completely differne . The bacteria don't have sets of chromosome that divide in the same way they do in eukaryotic cells so you have got a spinal apparatus and poles controls that occurring mammalian cels that don't occur in bacteria so we couldn't rely whole on a testing bacterium so we need to have a look in amamlian cells and one of the most common mutation tests is in the mouse lymphoma tests were it is looking at the thymidine kinase. Basically we start with a heterozygote plus minus thymidine kinase and what we are looking for is wether treatment with a chemical can treat that to TK minus.So the starting bacteria can synthesis TK the mutant bacteria cannot synthesis TK so if we put in a elective agent like triflurothymidine which thymine kinase enzyme will convert to a lethal form then we a have a simple genetic system in the presence of TFT the starter cells die and the mutant cells service so it a simple selective system. Now this si different from the ames test where thames test was a conversant mutation and as we described in one of the earlier slides you can see some very specific mainly point mutations and occasionally simple frameshift or a single base frameshift or deletion. Because the mouse lymphoma assay is a forward mutation system it is not a reverse mutation system. It can detect a much wider range of genetic changes. So not just point mutation but intrageneic deletions and allelic deletions, deletes associated with non-disjunction and recombination mutations and this shopping list is the range of genetic alterations that we find in tutors cells. So other people that say well id actually do this assay is much more relavent at predicting the changes that occur through forming a cancer cell because of the range of different genetic changes that it can pick up. To compare with the ames test be course this maximum somethignsystem can detect such a wide range of genetic events it has a higher spontaneouse mutation frequency. Theres more opportunities for spontaneous mutations to occur. So we don't need to treat as many cells, where as we normally treat about 100 million or 10^8 bacteria on an ames test plate. We only need to treat a few million mouse lymphoma cells at this concentration because of the spontaneous concentration inside. but one other important thing is is that these mouse lymphoma cells just like the ames test you have to allow the bacteria to grow long enough for the reverse mutation to allow the cells to synthesis histamine in the case of mouse lymphoma cells we have to allow the cells to grow long enough to get rid of the thymidine kinase. So those TK + - heterzygotes they start with some TK in the cell or within the cytoplasm. We hit them with a chemical and mutate them to TK - - homozygotes but that TK is still there. There not synthesising any new TK but they have go get rid of what is already there otherwise those cells will die in the presence of the selective agents. So we have to go through whats called an expression period when the TK - - genotype becomes fully expressed and there is no TK within the cells. Now that expression period requires several divisions a bit like the bacteria going through several division in the presence of histidine. That growth period between the initial DNA adamage which is the genotypic change and when we select for the phenotypic stage thats called the expression period. Now in bacteria thats very short because bacteria divide every

20 minutes. So that expression period is very short. Whats the expression time for a mammalian cell like this then? there about 15 hrs. So we have to go through a couple of days for the genotypic change to be expressed as a phenotypic change. Now because we have to subculturee the cells during that period we have to check that they don't get too dense and that the cultures don't stagnate and then we have to get into the selective medium and they have to grow in that. so the cell numbers are changing during this period.So we don't need to correct or calculate the immune frequency in the ames test we just count colonies on a plate. But here we are going to have to relate the mutant frequency or calculate the mutant frequency based on the viability. Because the cell numbers are changing and the whole growth period is much longer than it is in an ames test.So thats another difference. So again we test to high concentrations to 10 mili molar or 2mg per ml. We cant really handle ppt in these of cultures and it felly dose get in the way because you are subculturing and these cells grow in suspention, they don't grow as a monolayer on the plate. They have to grow in suspension. So washing out a ppt is really difficult.so we have to avoid ppt conditions but we need to know if we have gone high enough so normally we will include one insoluble concentration. For human pahrmacetuicals they actually lower the top concentration so its different from testing all other chemicals. We usually test four concentrations. We prefer duplicate treatments but there are labs that do single treatments and maybe they'll test more concetrations but again these cells don't have much in the ways of metabolic capabilities so we need to test with and without rat liver S9. But in ammalaina cells you cant treat for long periods with S9, this is because those oxygenses or the oxidative enzyme capasit is a problem. If you leave that int he precedes of the cells for too long then you get brake down of the lipid membranes and that produces reactive oxygen speeches that is toxic and causes DNA damage. So we cant treat for any longer than about 6 hrs in the presence of S9. It just becomes too toxic. So basically the protocol is to test for 6 hrs with and without \s9. We used to also include and addition longer 24hrs treatment in the absence of S9 but the latest recoemdations are going to go before the OECD coordinators in about three weeks time and they are suggeting that that is no longer needed and that that 24hrs treatment is no longer needed. There are some people that are worried about that and think that it should be incurded as it makes for a more supportive test. So we are going to have to see if thats going to be approved or not. Because your doing oto do subcultures in the cells because the cells are toic so you'll reduce the number of cells you have to engineer the test to make sue that at every step and at treatment through expression you are retaining enough cells to give at least 10 and preferably 100 mutants. Thats to make sure you avoid zeros, we do not like zeros in these experiments. So if you had zero mutants on your control plates or smoothing like that it really doesn't give you any indication of the sensitivity of the test or its ability to detect a mutation. So by making sure that we subculture and carry through enough cells to always contain the defined number of mutants then we make sure we avoid the zeros. We particularly want to avoid zeros not he controls plates and if we can we want to avoid zeros the treated plates because that might mean that there are so few surivign cells that we are missing or failing to detect the compounds effect.

MLA: Summary of Practical Procedure



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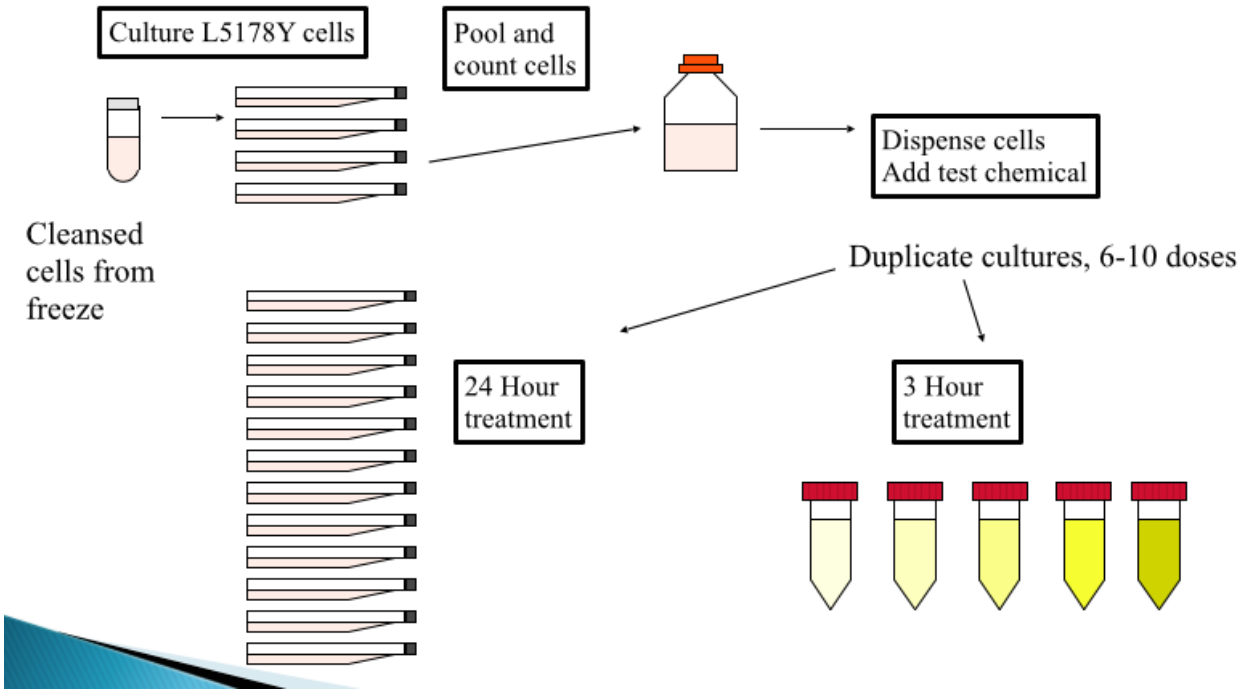
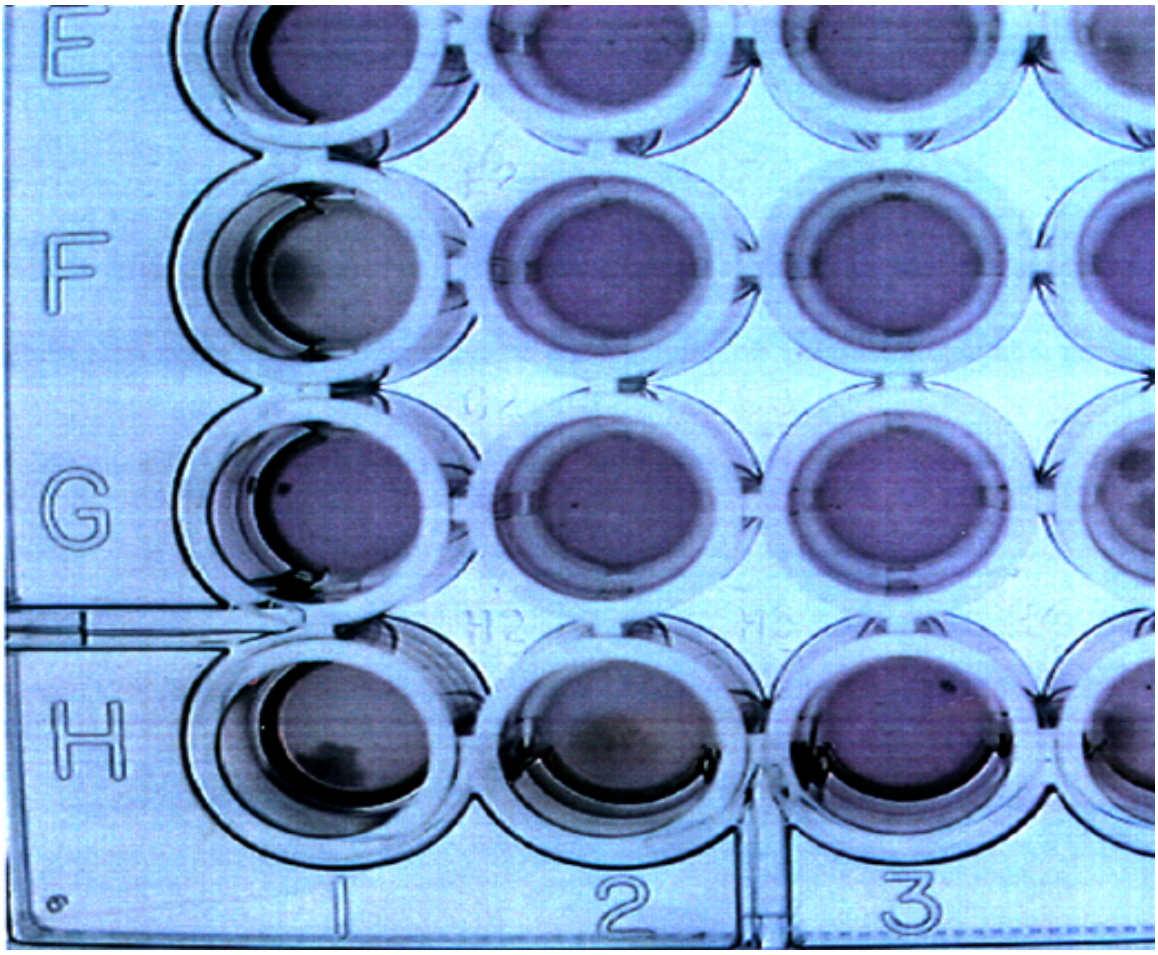
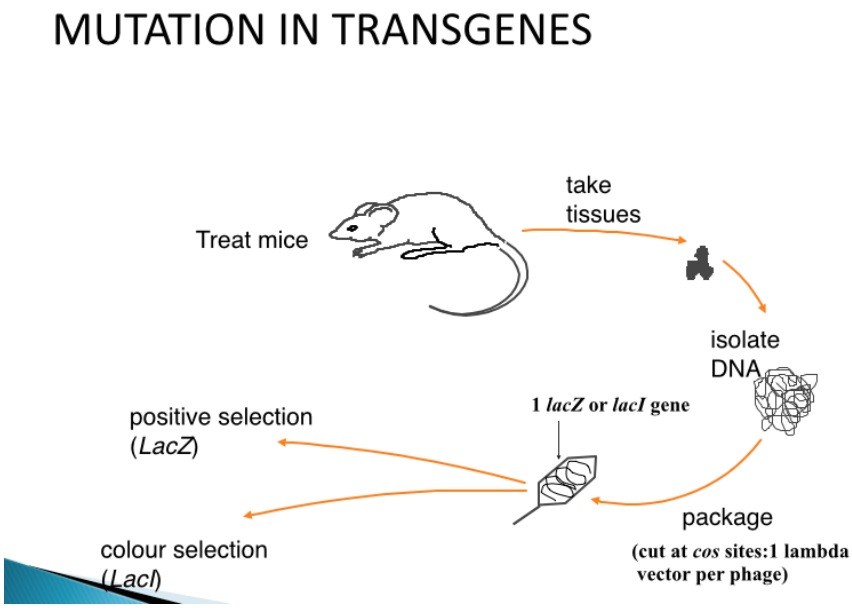


So just a couple of cartoons to for who this is conducted. we need to rapidly grow cells and they need to be cleansed pre existing mutants before we grow the cells. We grow them and count them and in short treatments we put them in tubes as because its only going to be for three hours if we are doing the mon treatments we put them in flasks and then after the treatment and wash off we express the two days then we count. We then plate for survival and we place like two cells per well and we also plate for in slection medium mutation plates where we will be plating them out 10^5 cells per well and these are then incubated for a bout fie days to allow the colonies to grow. Now we can do this, we used to always to this in the 96 well plates. But you can do this in petri dishes or in agar and in both cases you get, two different



types of mutants. You get some wells will contain a colony that is quite pale and maybe occupies a third to a half of the well after 12 days incubation and then in other wells you get small compact colonies. Now these have been analysed genticly and basically these larger colonies have quite small genetic changes or point mutation gene mutations or very small deletions. Techie are able to grow at a normal rate and normally dividing cells, whereas these cells that form the small colonies are firstly much slower growing which is why you have a small colony and they tend to contain much larger genetic damage so larger genetic region or rearrangements. So the types of colonies that we get can tell us something about whether not we are looking at a point mutation or a chromosomal change or chromosomal damage and that can be quite useful. So thats the mouse lymphoma assay, which is quite different from the ames test but ti dose give you different information.

So to finish of this first talk we are going to look at transgenic animals, now these have been around for a while but we have only had an OECD guideline on this test for about three or four years. It took while to get enough data together and as we mentioned earlier these animals are very expensive so you don't tend to get a lot of people publishing data on them. The two most commonly used transgenic animals are both have a target transgene associated with beta galacisade activity. The the case of LacZ that is the beta galacotosidease gene that in the case Lac I this is the promotor for the beta galacosidase gene and these transgress are located on a lambda bacteriophage vector which has been incorporated into he mice and there are multiple copies, in the case of LacZ ts 40 copies. concatenated head to tail in one location. So there are multiple copies of the transgene and again you have quite a large target. One interesting thin is that the transgene is neutral, so if the transgnee is mutated by treating these mice with a chemical it disant confer any advantage or disadvantage on the animal and that means there is no selection pressure. There is not selected for or against and its quite neutral and that is quite an advantage because it means you are looking at real absolute effects when looking at mutations in that transgene. So what we do is we treat the mice and we will go through this protocol in a minute. We extract DNA now the lambda bacteriophage vector has enough DNA there that when you take the DNA out of the mice you can add to it whats called a packaging mixture and this is a mixture of enzymes and portions that allows the lambda bacteriophage to reconstruct it self. So it basically takes all the bits. We add the enzymes and proteins and things like that and the DNA does the rest. So we put this stuff togather and we get reliable lambda bacteriophage. Now those lambda bacteriophage will contain the transgene. Some of which will be normal others of which will be mutated. We then test those bacteriophage on a sensitive strain of baitera. Its usually E. coli C and the bacteriophage of course kills the bacteria and forms plaques or clear areas on the plate. Depending on wether that transgene is normal i.e. expresses beta galactosidase or is mutant i.e. dosnet express beta galactosiadase then we can see the mutants form the non mutants by a colour reaction in the agar plates (blue white screening) So here is a cartoon of the transgene of a lambda transgene or the lacZ transgnee on the lambda bacteriophage vector. At the end of each insert are what are called Cos sites. These are important because they need to be intact when we extract the DNA and add it to the packaging mix, if those Cos sites have been destroyed then the lambda bacteriophage cannot constitutete or cannot reform.So if we are treating the animals with a test chemical that causes large deletions and it deletes through those cos sites then we may =wll have caused a mutation but we can t detect it because we cant get viable lambda bacteriophge out. So this test system is not very sensitive to chemicals that cause large deletions. It is very good at picking up chemicals that cause point mutation but chemicals that cause large



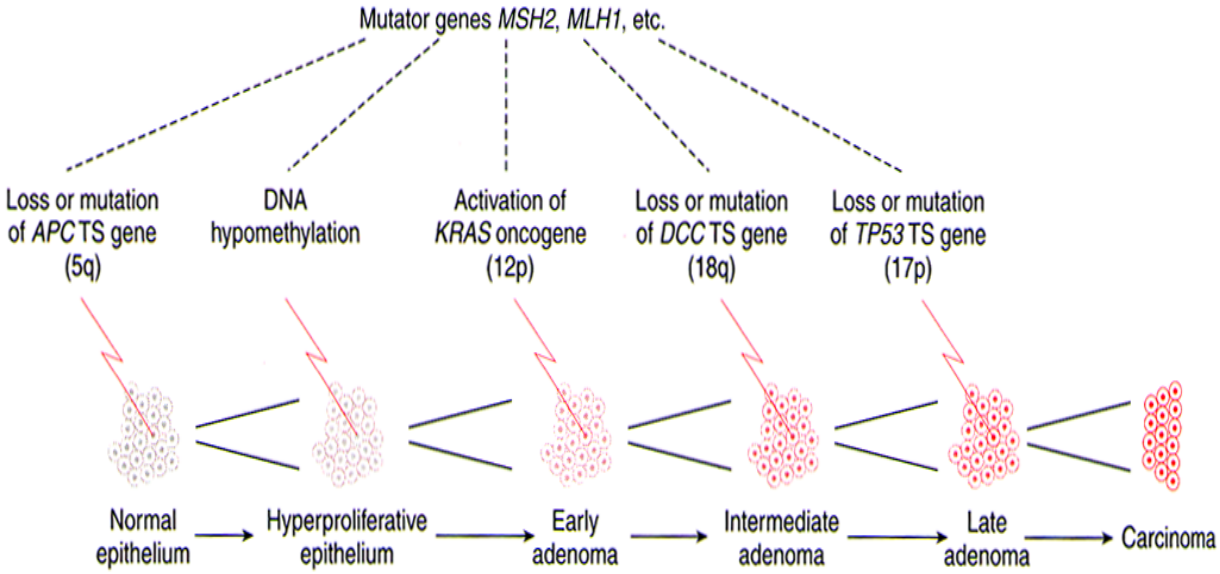
DNA deletions we might get Cos negative. So just to go through it again, once we have reformed our bacteriophagee they will lyse E. coli sensitive bacterial called to form plaques. We grow those on an agar containing a substrate that releases a colour or a blue colour if it is present. So mutant plaques form a blue colour and wild type plaques form a colourless so there easy to count and with the LacZ system its easy to count and use whats called a positive selection system which means that the wild type bacteriophage die before they can form a plaque and therefr we are ony scoring mutant plaques and that makes the whole experiment much more methodical because we need much fewer plates and equipment in the way of incubators and things like that. So its basically an ex vivo assay and this is just to show you that the mouse looks like a normal mouse it docent have two heads of anything. AAlthough the mouse is widley used and widely published systems are MutaMouse and Big Blue there is quite an interesting model that has been developed in japan that combines GPTdelta that is another enzyme based locus with a spinal mutation. This can detect larger deletions as it is not so susceptible to trans negatives and importantly there is also a rat model, so these are mainly a mouse system and of course rats are quite useful because in most industriess you have much more data in rats. So you know about the toxicity the levels of blood and things like that. So getting superting data is somewhat wasier than if you have got a rat model. There eis much less data published in that. Now some chemicals induce mutations quite quickly in these in vivo models and others take quite a while to get built up and because these are neutral mutations they don't disapear. So once you have gotten to a neutral platoit docent matter wether it takes three days of fifteen days. Once you are at a plato it stays there. So we don't have to worry about loosing mutants and therefore we can treat for a longer period so to cover all of the bases we generally treat fro 28 days and then sample about three days later. So faster acting mutants will produce mutations and then they will plato earlier but other compounds that need metabolism will have there effects later. The germ cell mutegens shows there is a suggestion now that we might now need to go even longer. We can detect mutations in early stage germ cels at 28 days but if we want to detect mutations in sperm for example we probably need to go out to 56 days or even longer. So this protocol might change in detecting mutations in germ tissue. so these atre quite expensive expressents, when your looking at £200 per mouse and you have y=to dose for 28 days and you have to take lots of DNA sample and do all of the ex vivo bacteriophage manipulations and so on you see how they can get expensive and time consuming experiments. But they do provide some quite valuable data. You need several dose levels in order to look for a dose response. You need enough animals per set per group in order to look for heterogeneity but the useful hing about these models is you can datect mutations in and tissue. and we mention that now because when we go to the other talk and walkabout one of the other in vivo test that have been widley used is much more limiting for us to be able to look for mutagenic or genotoxic effects in any tissue at site of the contact for example or GI tract or lungs. As well as liver kidney brainn whatver is it really useful. So fairly strait ofrwardfrom the technical point of view and only hard due to money and time, treat the animals or take the tissue. We can put those tissues in the freezer, we can take or we might say we are interested in the liver but we better take a bit of GI tract and a bit of kidney as well just in case we need it later on. We can put those tissues in the freezer. You can store these for up to five years and then still just extract the DNA from them mix with the packaging mix and then do the ex vivo mutation expertments. So isolate the DNA add the packaging mix get that in the bacteriophage and plate on to to E. coli C and use the positive selection system in mute mouse to reduce the number of plates or a

recover selection system with Lac I. So thats the transgenic mutation models. So yes just because it causes a reaction in a mouse or a rat dosnet mean it will be the same in a human but if you intact compare rats with mice so lets say you are concerned about the ability of a chemical to cause a cancer in the rat so you did your test in the mouse. The mouses then trying to predict what happens in the rat ok. That is only accurate to 70% and thats between rats and mice. So the difference between rodent and humans is probably going to be even bigger. Now history tells us that there art chemicals that we believe are carcinogenic to humans and there are organisations like IARC (the international agency for Research on Cancer) lists 106 chemical that are considered to be human carcinogens. They are all rodent carcinogens so the rodent isn't missing anything that we are aware of that would be a human hazard but it has been probably been giving positive results with a lot of chemicals that are not human carcinogens. Meaning that they are over predicting. The regulators and health authorities would rather have it that way round though. They would rather the animals be more sensitive than the humans because then you not letting anything through the net. It causes problems for industry because it means they could be getting positive results in rodent cancer studies that are not indicative of a human and if they want to progress with that compound then they are going to have to do a lot of work to demonstrate that its not relative for humans. So for sure there are differences but if you like the differences are on the safe side. There has been a lot of follow up work on churnbel and in japan in nagusaki and heroshima for example. For sure there were increased frequencies of leukaemia and thyroid cancer due to increased radioactive exposure and there were reproductive effects like fertility and so on but we have not heard any body speak on this in quite a while but the impression that was given is that it is not as dramaic as everybody hears. Humans are pretty good at recognising damage and in particular in the reproductive process. If a feotus is damaged then humans are good at recognising it before that feotus develops. And spontaniouse abortion within the first month is normally the result of that and then we are unaware that there was any thing abnormal embryo. So ye there are lots of things to consider and humans are pretty reslant to a lot of these environmental exposures. Then having said that we worry about the fact that there seem to be so many more allergies now than 40 years ago.\Is that because we have been exposed to so many synthetic chemicals that what we used to be? is that more of an issue in terms of health in terms of cancer or genetic disease or is genetics involved. Is it genetics that leading to the increased number of allergies. Is it up to the pharmaceuticals to continue with the progression of a test even if they get a positive result in one of these test? usually its going to depend on three things. 1) do they have a backup compound that is going to be safe? Big pharma will usually be looking at a bunch of chemicals all at once that have subtle diffenreces, so they may have a back up where a small company may not. 2) what ar the economics is this drug going to treat a large number of patients or a wide spread peopleation so that the retrns are going to worth the aditional effort to resolve the extra effcrt to look for ways around the deskmanting results, if your looking at a very small patient population then your not going to sell them any until of the drug then its probably not going to be economical. 3) what is the medical need and are there any effective treatments already out there, because the regulator may well accept a number of unansewerd safety questions if the medical need is dramatic enough. So for example in the last year is=f someone had come along with an untested Ebola cure then people would have said well theres a 70% chance these people are going to die we don't care wether it might give them a mutation. If its going to save there lives now then we will try it. This is the situation that we were in with aids drugs a number of years

ago where the regulators they didn't care or not if those treatments caused cancer or not and many of them did because the nucleoside analogs which are antivirals did cause mutations. Now because so many people are surviveing aids and the treatment of aid sand there living longer. Now the health authorities are concerned about the areatments for aids and wether or not the drugs that treat aids might subsequently cause mutations. So it wasn't a cause for concern many years ago because it was a considerably un met need. It now is a concern because people ar living the disease longer. So the consideration you have to take into account is where each one needs to be taken case by case and askd these questions of do we have a back up? who do the economics look? and what is the medical need? thats what your going to cause your dissicion on? Is it worth it or isn't it? generally the pahrmacetical companies will make these dissections themselves because they look much better and they are seen in a much better light if they go to the regaltor authorities with a coherant story. So you don't want to go to the regulators with these results and say we don't know what to do about it, because thats not a strong place to be in and the regulators might tell you that they want you to change the world before they accept it. Its much better if you can go as a company to the regulators with a coherent story and a blanked risk assessment and a reason for why that drug should be safe for those patients in that treatment period in that age group, whatever. Its better if they take the dissections and do the work first. Right we will look at the last slide where at the beginning david mentioned he spends a lot of his time heloing clients try in to sort out those situations where etheyhave some sort of positive or negative results and they are trying to tease the real positives forth false positives and the real negatives form the false negatives. One of the big problems is that once you do a test and think abc to the bar charts that we saw is that what criteria do you use to call something positive? And across the literature and across the years there are lots of different ways that people approche it and every body got there favourites. You might use statustical significance, thats probably a good thing for some of the mammalian cells tests but its very sensitive for small increase for example in the Ames test. You might want to use a full increase over your control. You might want to look at your historical control rate and say well something is only positive if it goes out side the historical conrole rate that we have built up over a period of time. Thats good for a low frequency event but probably not so good for things like the Ames test or the mouse lymphoma assay. You might set a preset levels for the mouse lymphoma assay we had a working group that collected data from about 200 labs and they established a control distributetion and said well alright well we need see a umant freequency of X. So if the normal is Y then that diffenree btween Y and X is a mathematical increase that we would never normally expect to see within the controls population so we will put this preset bar where they say anything grater or increased beyondd this point has to be biological significant. They call this the global evalutaion factor. This si the only test system where we know of that this happens. It might however come in to others. There is then dose response where people are more convinced that if people see a dose reponce across several concnetration rather than at a single point. At a single point they don't know what it means and it may be due to toxicity at the highest dose or in the middle of the dose range. What is it telling us and reproducaibility. We have mentioned that now some o the regulators are only doing a single experiment. but most people feel much more comfortable knowing they have seen either a positive or a negative that is reportducible. It takes out one of the elements of chance that any experiment can give you a diffenret result on different day, even using the same compounds the same technicians the same glass and plastic wear because these are biological systems

and they have inbuilt heterogeneity. So these are problems for interpreting results for any body and they are pretty much every body has there choices and there all doing differ ones.

We are just going to spend five minutes introducing the next lecture so that we can scoot through it. So the agin just shows that with in the cancer process there are several step where ells of genetic material is important and so chromsomal damage and thats what this next talk is about (DNA damage, Chromosome damage) now this is distinct form mutations. The chromosome damages do play a significant t role in the



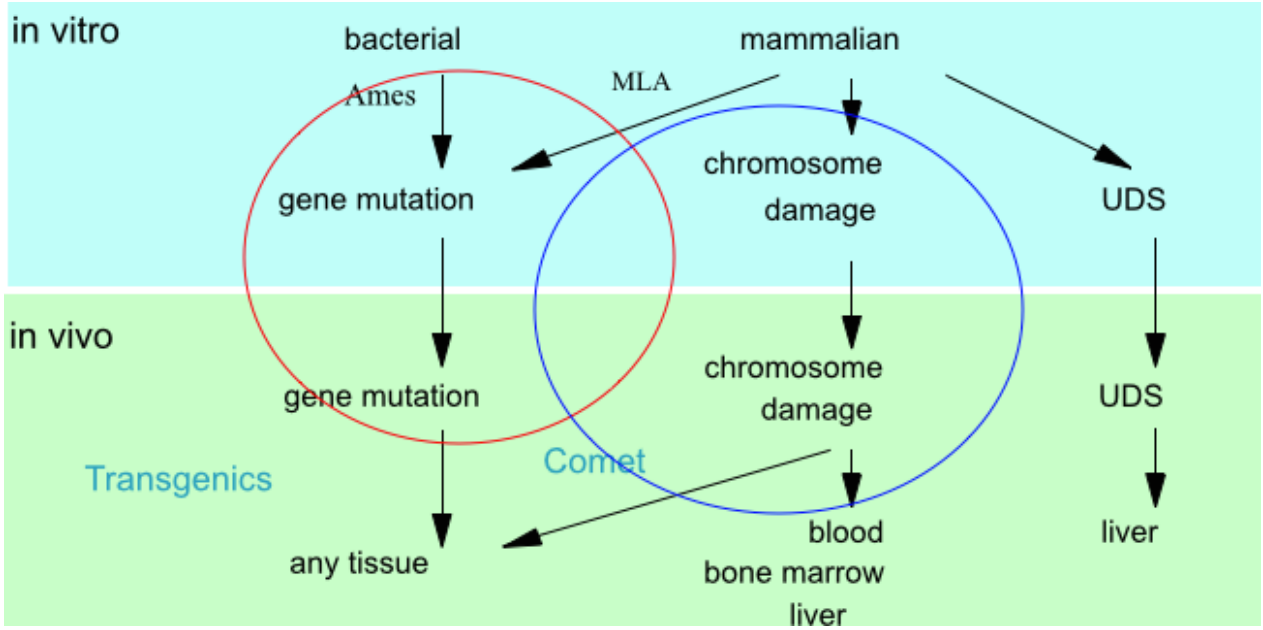
tumor changes, through initiation progression and inheriting diseases. Mutations and other events including chromosome damage generally lead to something that we put int he umbrella term genomicsinstability. This includes a raft of things like chromasome loss or aneuploidy, abnormal chromosome segregation, deletion of part of the chromosome or recombination between chromosomes, these are all typical of unstable genes. This is why when you look at cancer cells you se lots of genetic changes, those are changes that have occurred as the cancer has grown. They are not what cause the initiation event they are what has happened as a result of the uncontrolled rate of proliferation. Once a cell becomes mutated and once it has experienced some genetic change then the whole genome seems to become unstable and you get a build up of these other kinds of changes. Genomic instability is associated with an increased cancer risk. The best examples that we have are Blooms syndrome, colonic carcinomas and retinoblastoma. These are familial so there is a genetic basis associated with genetic instability but more startling perhaps is that there more thousands of genetic disorders that exist and many of these are at a very low frequency and you wont have heard of some of them but you will have heard of the most common one which is down syndrome. Which is an aditional chromosome 21 there are three copies rather than two. Some of these others we might of heard of are seen below:

>4000 described. Some are manifest at birth, e.g.:
Down's syndrome (trisomy 21)
Patau's syndrome (trisomy 13)
Edward's syndrome (trisomy 18)
Klinefelter's syndrome (XXY)
Turner's syndrome (XO)
These are all manifested at birth so these are numerical chromosomal changes that are occurring in the reproductive process.

Others manifest themselves later, e.g.
Duchenne muscular dystrophy (childhood
hypogonadism (adolescence)
Huntington's chorea, polycystic kidney disease,,Alzheimer's disease (later in life)

Others only appear later on in life such as huntingtons. So a lot of disease conditions can be associated with chromsomal change. Even more starteling and we are not going to read all of these. You can look at them later. It is estimated, and this goes back to what we were saying a while ago that humans seem to be pretty good at treognising a genetic abnormality and probably report it at the first month of pregnancy.So we may often not know that even a women is pregnant. Based on all of the datas that we have got we have estimated that 8% of all our embryos formed are chromosomal abnormal. Most of those will not survive and most of those will be aborted but as you saw on the previous slide that some do get through a viable birth. So chromosomal damage is important and as we mentioned in the earlier talks we are not just measuring gene mutations which is what the proviso talk was about but we are also need to look at structural and numerical chromes damage because its important. Not just for the cancer process but for other aspects of health so we have test that we can do in vitro for chromasomal aberrations or micronucleus and we can include and just get a way with two test to minimum the effort then an ames test and an in vitro micro nuclease test will pick up both structural and numerical change. As we mentioned before high concentrations and extreme conditions and the need for metabolism. In vivo we can look for chromosome dam but only in limited tissues. We can only really look in bone marrow and blood and we can measure the micronuclei in the liver. So unlike the transgenic mutation assay where we can look at any tissue if we are looking specifically for chromosomal damage then we are limited to only a few tissues and that can be a problem because we need a seagate assay and thats what we will come on to last this morining. We can looka for DNA damage most, chromosomal aberrations result form a double strand DNA brake and we can look for those brakes directly using whats called a comet assay and that we can do in any tissue. So we can look directly for chromosomal damage we cant look directly for micronuclei in any tissue but we can look at the precursor DNA double strand brakes in the in any tissue. So thats what this talk is going to be about. So if we look across in vivo and in vitor we can get gene mutations in vitro and in vivo which we have explained in the previous talk. We can look for chromosomal drage in vitro and in vivo but in vivo it is only in limited tissues and so we have the comment assay thattells us about DNA strand brakes that we can do in any tissue. So is really just trying to square the circle in terms of looking at all of the relevant end points in both invert and in vivo. So we

have already mentioned the important of doing that so we are going to describe three types of assay looking directly for structural chromosome damage looking directly for micronucleus induction and looking for DNA strains brakes.



For structural chromasome damage we can measure this in cultured cells in vitro and in certain tissue, generally bone marrow or blood in vivo. What were doing is actually looking at the chromosomes when they are visible so thats when there in the metaphase stage of mitosis or meiosis. So the cells have to be dividing and this is what limits us in terms of the number of tissue we can look at in the whole animal because getting dividing cells is not easy form a tissue like liver or kidney or something of that sort. The bone marrow and there for the cells which are produces in to the blood or the white cells within cells within the blood we can make them divide after treatment we can take blood and stimulate the white cells to divide by using a mitogen like phytohaemagglutinin, that will stimulate the white cells to divide. Then we can look at the chromosomes. Now the problem with scoring chromosomal aberrations is that normal chromosomes at metaphase can take on a number of different appearance. They can look quite different just form one cell to another. So it requitress quite a lot of extensive training, its a bit like pathology but pathologist has to spend a lot of time looking at the way normal cells appear in normal tissues before they can diagnose abnormal cells. Or abnormal tissues and its the same with metaphasee tissue. Before you are aware of all of the different ways that normal chromosome can appear then you cant really identify how abnormal chromosomes. So We will look at some pictures in a minute and it will illustrate that. So basically the cell types that we use fro chromosomal damage. In vitro we can take normal lymphocytes and whole blood. We can stimulate the cells to divide because within the blood they are in a resting stage called called :G0 and we can stimulate those cells to divide by treatment with a mitogen or we can use established cell lines. Most often these are from chinese cell hamsters and that useful because they have only got 20 odd chromosomes so there easy to count. V79, CHO, CHL are the most common . V79 come form lung the CHO are overy and the CHL are also lung. These cells are

imortal and they grow in exponentially but in becoming mortal they have already become chromosomally rearranged. Non of thee cell lines are truly diploid they have already undergone some chromosomal rearangments. If you remember what we said before the brake. Once a cell looses some of its integrity. Once it starts to become genetically abnormal it tends to become genetically unstable. So further changes occur randomly and spontaneously and that can cause a problem. Also all of these three most commonly used Chinese hamster cell lines are p53 deficient. So if a cell is p53 deficient then it can experience DNA alterations but it wont recognise them and therefore it wont go into apoptosis. So cells that should be dyeing because of the DNA damage carry on dividing and we see chromosome damage in cells that should be dead. Because there p53 defiecient. and that can lead to misleading positive results as those cells should be dead and there no. So the latest OECD guidelines state that we are most likely to get reliable results if we use p53 competentn human cells such as normal lymphocytes or as we will se when we come to look at human nuclei there is a nice human cell line called TK6 which is p53 competent and thats really quite useful for doing micronucleuse test. In vivo we have to use cells that can divide so bone marrow cells and spermatagonial cells or we can take blood form treated animals and we can stimulate those sites ex vivo and we can look at chromaomal damage in them. The normal test protocol is quite similar to that for the mouse lymphoma assay that we discuss before. So the presence and absence S9. We cant treat for longer than 6 hours because of the toxicity of the S9 but in the chromosome test we allmost always include a prolongedd treatment between 20-24 hrs so around a bout one an a half cell cycles in the absence of S9. This is to pick up the sort of compounds that need to be present for a whole cell cycle in order to produce there effects. Classic examples of this are nucleoside analogs things like 5-flurouracil and things like xemothymadine need to be present during a division cycle and they need to be present when the DNA is replicating because thats where there going to be incorporated into DNA and thats when there going to porduce there effects. If you only do a short treatment like for 6 hrs then you might miss that. We sample one and a half cell cycles after the start of treatment which is to allow for any cell cycle delay if the cells pick up mage and try to repair it. In cerium cirumstanceswe may actually include a sample time which is 24 hrs later than one and a half cell cycles . So it could be sampling at 20- 24 and 48 hrs to pick up andy delayed effects. As with the other mammalian cell assays. A number of concnetrations preferably due to duplicate cultures but you can use single replicates s long as you score the same total number of cells. Now we mentioned earlier this morning that we don't like zeros in these tests. So to avoid zero counts in the control cultures OECD have recally increased the number of cells scored to 300 cells per concnetration. That usually will mean that your getting real positive numbers of aberrant cells in you control cultures so you can establish the statistical power of the test. Again we are testing to extreme levels where we are going to high concntrations for eveything accept human pharmaceuticals where they are lower (no ones asked me why they do that by the way). So why is it lower for human pharmaceuticals than for any other type of chemical, ITs because we have much much more safety data and in most cases we will have carinogenicity data so the regulators are less worried about pickingg up absolutely everything in the gene to test. This gives you enough data to be conformtable that you are not harming healthy volenteers and patients in the clinical trials. By the time it gets on to market you are going to have a cancer study so if there were any genotoxic effect then it would be picked up there. so they have pushed the limit doesn to allow more compounds to get into clinical trials easily. So we are testing to high concentrations and we

are testing to relativly high levels of toxicity. We need to see around 50% toxicity in these experience to make sure that we ahvnt missed any thing. By getting into these levels of toxicity you are overwhelming the defence mechanisms of the cell your therefore staing a better chance of any true genetic effects coming through. But you can introduce misleading results and artefacts or misleading positives as result of the toxicity thats occurring in these extreme conditions. So this is ban obvious select picture of a human lymphocyte chromosome prep. All of the chromosome are nicely separated and easy to sore and all of the chromaome are nicely arranged and there not a lot in the way of kinks or bends or anything like

that.

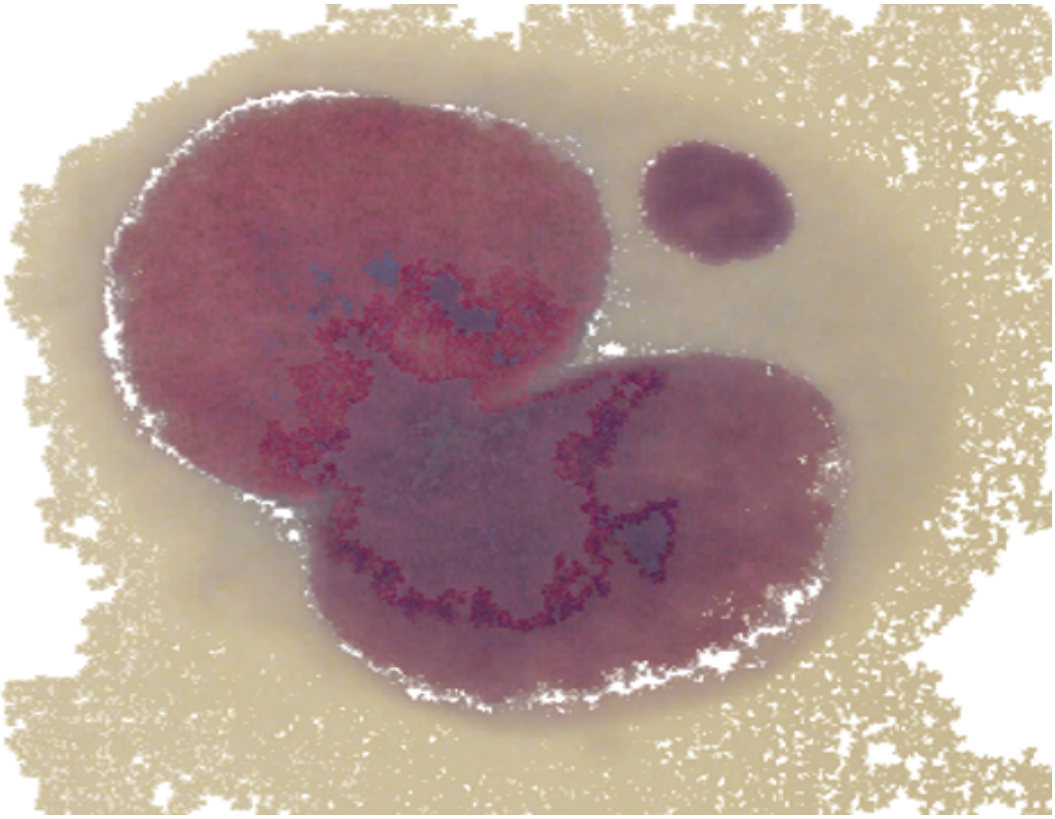
At 1 we have a complete chromatid interchange and at 2 we have an incomplete chromatid interchange. So these would be the sorts of aberrations that you would look for. we are going to come back to this picture in minute. This is another human lymphocyte cell on the right but look now at the difference in the way the chromasomes appear and you will see what david means about the important of training. It wouldn't be to difficult to pick up is an aberration in this cell. But in this one the chromatids are much more kinked so are there brakes or is the 1 a brake because it loos out of line . What we have here is a double chromatids isochromatic fragment or a deletion. So just think forwards to what will happen to this fragment and hat will happen to these interchanges when the cell tries to divide. So we have stopped these cells at this point so that we could view the chromesomes but if we hadn't done that then we would have allowed those cells to go through compleat division and go through compleat interphase. What would happen to these exchange figures and the fragment in the other cell? Well these might go to one daughter cell but they cant spotit and go to both because of the rearrangement. The same is true with the fargment because the fragment in this preperation. It hasn't got a centromere so it can tattahcto the spinal so it might by chance end up in one of the daughter cells or daughter nuclei, it might not. So there is a potential that this fragment or these exchange figres just get lost and do not appear in either of the daughter nuclei. You'll see the relevance of that in a minute. Just keep that in mind. So thats how we measure chromasomal mage in vitroo. In vivo we can do this in bone marrow because bone marrow cells are dividing and we usually do this as an accute study. We can do a single administration and sample at two time points but this uses more animals than doing two administrations and sampling at a single time

point. Around 12-18 hrs after the last dose around one cell cycle after the last dose. Because we have done two administrations we are allowing for cell cycle delay. Usually five groups of animals per set per group we can do the study in only one sex and therefor reduce the numbers of animals.if there are clearly no sex diffenreces. Again several dose levels and we need to be scoring quite a lot of cell. This has recently been increased, it has been doubled and has been in the latest OECD guydlines again to avoid zeros. So we are not getting zero aberration counts in controls. So this si pretty demandding and you think of the training that goes in to understand all of the efferent ways that normal cells can appear and then you have to score 200 cells per animal, 5 animals per group an d 100 cells per dose group. Wearing down the microscope to see if there are any brakes or fragments or rearangments. It is very demand ing and very time consuming. In these in vivo test we usually also take blood sampples and analyse the amount of test chemiccal in the blood. Theses important because if we get a negative result after scoring all of these cells.We need to know that the bone marrow ws exposed. Now there are some compounds that you would administer orally and they would go strait through the GI tract.Come strait out in the fees and nothing gets into the systemic circulation and therefor the bone marrow and the target tissue wouldn't be exposed. So its not suprising that you get a negative result. The regulators requre that we have some prooftha the bone marrow and the systemic circulation did contain the test chemical or its metabolites. otherwise a test chemical is not worth the paper its written on. This can be quite demanding for indstry like the chemical indoorsry the food industry. They don't automatically have bioinformatic methods that allow them to measure test chemicals in biological fluids like blood plasme. So that can be quite semadinging and quite expensive just to get that bit of the equation right and if we do measure these concentrations then we are particlery if we think that we might have a no effect levels or if we think that we might have a threshold effect and there is a safe level of exposure that we can compare those concentrations in the the animals to humans concentrations in normal use and in order to do a risk assessment. Questions on chromap's?

How do they test it in the blood, do thy use flow cytometry or elisa or something? not for structural aberrations like this no, micronuclei which we will look at next yes, but for chromasomal aberrations no machines accept a metaphase finder which saves the time of scanning the sides for suitable preparations and there are image analyisi software that will try to tel yu have or not a normal karyotype or an abnormal karyotype but to my knowledge every one of those needs human intervention. A human microscopist or cytogenetacycts would have to go back and check wether the machine has identified an aberrant chromosome or not. So you can use image analysis to save some time but for structural chromosome damage, we are not aware that there is an image analysis software out there that can do totally automated scoring with 100% reliability without any human intervention.

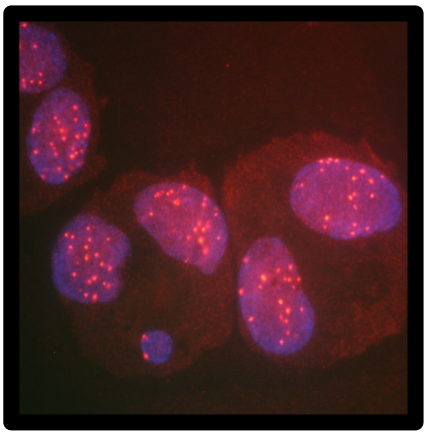
Which is why its so demanding and which is why the micronucleus test which we are going to talk about next has become so popular. These kind of rearranged chromosome and fragments like this may not get incorporated into the daughter nuclei after division at all and if there not then they can end up as a fragment separate from the two daughter nuclei, get wrapped up in a little bit of nuclear membrane and therefor they can look like a micro or small nucleus, we can see these in the interphase cells so after a cell has gone through cell division into the next interphase thats where we would pick up these fragments or

rearrangements, whole chromsomes as micronuclei.We can do this again in vitro of in vivo using the same cells or the sam tissue. Again though we have to have cells that have divided. We cant odo this in non dividing cells but micronucleiar much quicker and easier to score. Docent requiree the same amount of training, its much easier to automate and because of that we can score many more cells per sample so we can increase the statistical power of the test. It is important that we know the cells have divided other wise a negative result or absence of micronuclei isn't telling us anything. It might just be that the cells haven't divides we are looking at interphase nucle. Cells with an interphase nucleus we don't know wether they have divided or not. So one of the favooured ways to approach this is to use little trick. We add a chemical called cycokilasin B which blocks cytoplasmic division. So it alowsnuclear division but lbocks cytoplasmic division there fore we end up with bi nucleated cells. If we can see binucleate cells we know they have divided. So that gives us a target population that we know hase divided in the presencee of after the treatment with the chemical and thats important. Again we can use all of the different cells types that are used for chromsomal aberrations but p53 competent human cells ar preferred. So it could be for example french blood cultures air it could be whats becoming quite common ar these human lymphobloid TK cells. These ar not tumor cells. they are cells that have been imortalised by treatment by eps tine barr virus. So they ar not tumor cells as some of the other tumor cell lines that are around like HEpG2 or Hella.Those are derived form tumours. These are not derived form tumours, they are human lymphablasotids stem cells that have been made mortal by treatment with eps tine barr virus. Similar sort of protocol to the chromosome like the aberration test in terms of numbers of treatments for example. But we sample a little bit later. We need get the cels past that mitosis and into the next interphase. so instead of one to one an a half cell ccyles after the treatment , we are going to sample one and a half t to two samples after the starter treatment. So this si a binucleate cell seen below:



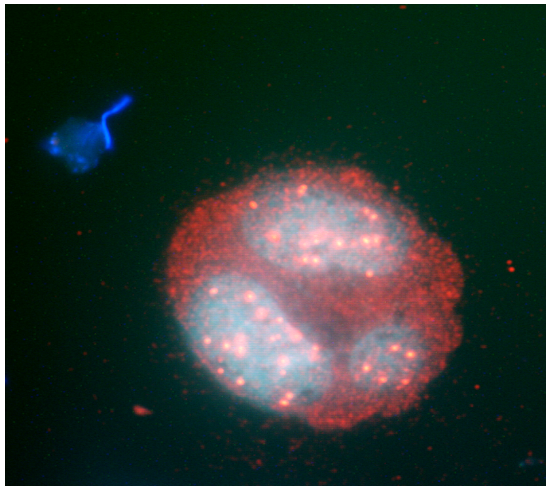
With a micronucleus. as we said there is a similar sort of protocol to the chroma's with a number of concentrationspreferable duplicate cultures but you can do them in single cultures as long as you score the sam total number of cells. If you do use single cultures its better to use more concentration so you can see the dose response curve. We have routinely scored two thousand cells, so this is ten times more for chtromasomal aberrations. 2000 cells per concentration done give us lots of zeros so its not been increased in the latestsOECD revisions. Again we need to get to around 50% toxicity with concentrations for around the same as the chromabs. now we mention that we can get micronuclei both form fragments that haven't got a centormere and from whole chromosomes that have failed to attach to the spindle for some reason. That failure to attach might be because we have had a rearrangement like those chromatid exchanges or it might be because the spinal itself has been damaged. And those micronuclei will contain centromeres and we can distingue between micro nucleui that contain fragment sand micronuclei that contain whole chromosomes by using a pan centromeric probe.Now why is this iortant. Because if the micronuclei contain whole chromosomes and if that it=s a result of spindle damage then the chemical is not attacking the DNA, its attacking the spindal. ITs a non DNA target. The default interpretation of a DNA reactive genotoxin is that there is no threshold. Theres no safe lev. We actually now know that there are safe examples but the defualt assumption that there is no threshold. Wherasif the chemical is damaging a non DNA target or if its damaging the spinal then it will have a threshold and thats important in terms of risk asseesment wether or not we can establish a safe level of exposure that humans can experience. So this si just reiterating what we have already said. The default position is that genotoxic agents of genotoxic carcinogens do not have a threshol. This really comes form tradition biology where erudition gives you linear dose response curve that tracts right back to the origin. Its not surprising really becuse radiation dose not need a transport system to get into the cell it dosent need metabolism, it cant be detoxified wherease chemicals ned to get into a cell so thats a barrier, they often need to be metabolised to produce a reactive metabolite. So thats neuter barrier there for there are processes that have thresholds associated with them but the default posttion is that is assume anything that damages DNA is like radiation and it dosnet have a threshold. So you have got to proove that your chemical has a threshold and therefor e it is safe for human exposure if you possibly can. so for chemicals that damge a non DNA target we treat them the same as we would a toxic substance or a non genitoric carcinogen that is there will be a no effect level. Somwhere below that level then it will be safe to expose human beings becuase your not going to see any genotoxicx effects low the level at which you see toxic effects. So if we find miconucli that are centromere positive so its an effect on the spinal then its important to establish a non effect level but micronucleii are only one manifestation of aneuploidy. We can get chromosomes that distribute unequally. If there is damage then there is some damage to the spindle we can get chromosomes that distribut euneuqlay to the daughter nuclei .So if its a human cells there are 47 chromsome sin one nucleus and 45 in the other. There are no micronucelues. None of those chromes have been completely lost. But there is an unequal distribution and that is till a health problem. How do we pick that up? that is called non-disjunction when you get an uniqueal distibusion. How do we pick that up? could that occur at concentrations lower than we see micro nuclei. Because if it dose then the no effect level for non disjunction is more critical and is more snesitive than the no effect level of the micronuclei. So in terms of establishing safety we need the most sensitive measure of aneuploidy and

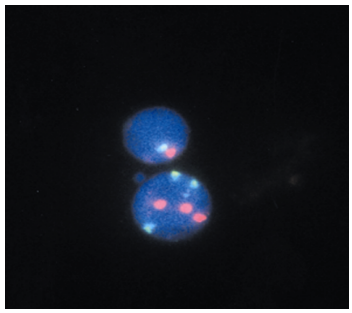
- 3 stage process
  - *In vitro* micronucleus test
  - Pan centromeric probe
  - chromosome specific probing (non-disjunction)
- Identification of threshold values ("safelevel")



Jake Ireland

that would be non-disjunction. We tend to think of the spindle as being a bit like the cable son suspension bridge. Those cables on the bridge are not single pieces of metal they are made up of multiple fibres and the spinal is made up of multile fibres. You could go along to a suspection bridge with a hack saw and you could cut through one or two of the strands and the bridge would no fal dow. But cut through enough of them and it will and its the same with the spindal. You can induce a certain amount of damage or slight damage to the spindle and there will be no consiquence as there are no micronuclei. You could induce a bit more dmage and some chromosomes will fail to attach to both sides of the spindal and will segregate abnormally giving you your unbalanced chromsome distrabution. Like chromosome 47 45. So non disjunction will occur where there is some damage to the spindle but not enoughdmage to lead to chromesome loss. That will occur at a higher concertion. Thats where we get micronuclei and if we go even higher then we creat so much mage that everything just falls apart, the cells stop dividing and then the micronucleus level come downbecuase theres nothing going into the next interpahse. they are all dyeing at mitosis because they cant comleate divsion. So the important question is we can establish a no effect level for micronuclie and we can establish a no effect level for non-disjunction but how big is that diffenrecance and is it important. If we are going to measure non-disjunction then we need to use a chromosome specific probe and not a pan centromeric probe. This is pan centromeric probing so lots and lots of suppressant signals within the cells. Thats no good for telling us about unequal disbarstion of chromsomes. hers another one includeing micronecluis with with pan centromeric probe. We need to lookat whole chromasomeprobes,s o this si a binucleate cells treatd with cultureseen it think. wE probed here for chromosomes 1 and 8 and this is a human lymphocyte. You see there should be two green and two red signals if the chromsomes have sgeragated equally and we have got a doubble non-disjunction. so this is how tyou measure non-disjunction you use whole chromosome probes for two or three chromsomes. You prob anymore than three and it becomes messy and you cant score it. But that iceves you enough that gives you a sensetive measure of non-disjunction. So thats how we can measure non-dijunction. How important is it that we measure it well we now have g=quite allot more data and people have done non-disjunctionexperimnets and compared them with micronucei expermients and the difference between the no effect level for micrnuclei and the non effect level for non-disjunction is no more than a factor of two. Its really much closer togateher than we though it would be.So what this means is that if your developing a product you do your micronucleus test. You show by pancentromeric probing that there mainly centromere positive so they have an anueploi effect and there will be a threshold. you can establish a no effect level and you can compare that no effect else with your human exposure. If that margin is very big then you dont need to

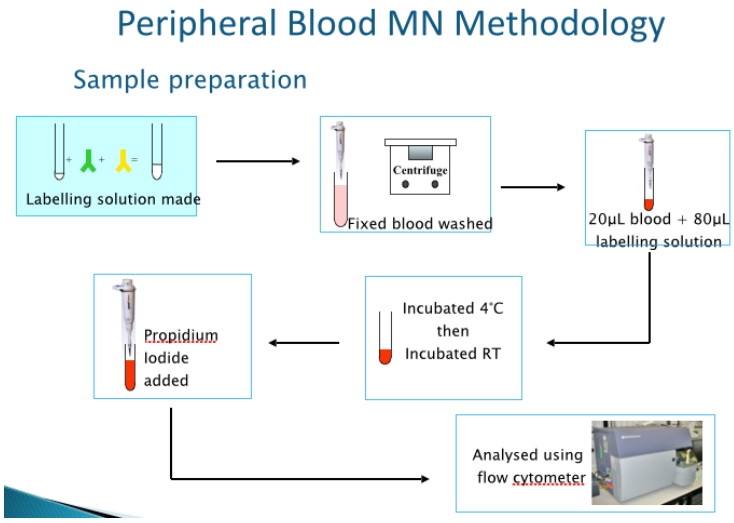




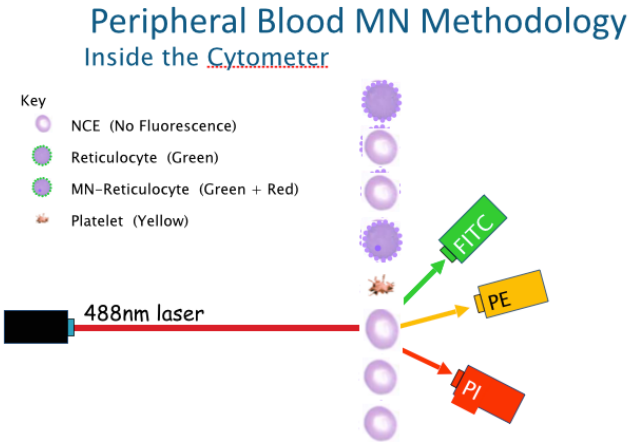
worry bout the fact that the no effect levels for non-disjunction may be two fold lower. IF this is 1000 fold and it would be 500 fold here it is still massive and nobady cares. But if your looking at smalll margins and if this is only ten fold and therefor your non sidjunction non-effect level may only be fivefold then its going to get an accurate figre. So on occasions even if we have centromere positive micornuclei we have got an anewgen , we can establish safety margins. We may have to go on and do these non-disjunction tests. To get a more accurate measure of the safty margin. Thats micronuclei in vitro.

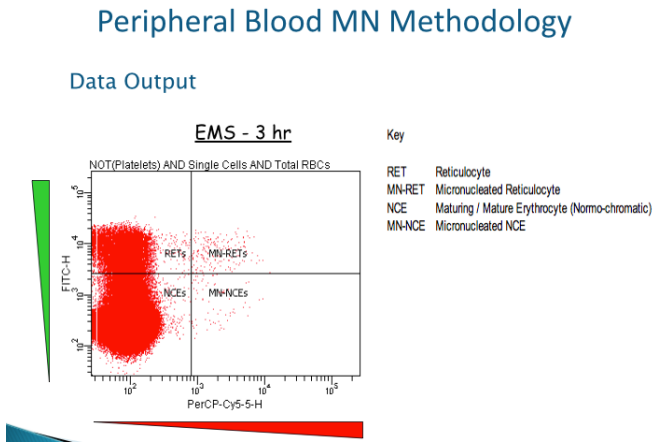
Ok so we can do pretty much the same in vivo.but in vivo we tend to do it in erythrocyte precursor cells any idea why? They don't have uncle which makes it real easy.Sop we are not having to search for a micronucleus in a cell that contain sone or two neucli. The erythrocyte once there pushed out in to th bone marrow ar young erythrocyte they loose there main nucleus. So if a micr nucleus is formed like a fragment or a whole chromosome is formed during the last nuclear division that micronucleus is left in the erythrocyte when the main nucleus is extruded. So its real easy to score. We can also meahure micronuclei in blood. Those young erythrocyte which are called reticulacytes as they sitl contain RNA until they become fully mature and that RNA is used to synthesis all of the haemoglobin and porphyrins and such that are needed for a mature red blood cell. Those reticulocytes we can score micronuclei in them in the blood. So we can score both in bone marrow when the cells are first formed and in the young reticulum when there pushed out into the blood. We can do the same centromere probing as we did in vitro to look for whole chromasomes to look for aneuploidy. So when the cells divide if there s damage then it will form a micronucleus when the nuclei are extruded. Then that micronucleus gets left behind and we can pick it up in the immature erythrocyte with in the bone marrow or in the blood. So we tend to use these days a DNA specific stain like acridine orange. So these big yellow blobs are the nucleated cells. Here are your immature erythrocyte which are red because we do a counterstain with primidinan iodide which has got or will stain the RNA. You probably cant see them but there are some really dark almost ghost cells, these are the mature erythrocyte and there wont be many of them in the bone marrow because the maturity mainly happens within the blood so we quite easily distinguish the young erythrocyte and the micronuclei using a stain like acridine orange. So typical study design for a bone marrow test. We can do one administration with two sampling times but this uses twice as many animals so more common is to do two daily administratiosn and sample 24 hrs after the second dose. We can recombine this micronuclus test with the comment assay. Which we will come onto in a minute and for that we would need to three administrations. This is to incorporate the optimum sampling time for the comment assay so we dose at 0, 24 and 45 hrs and sample three hours later which is what we need to do for the comment assay. So we can combine two end point to a single set of animals which is goo. We can also do bone marrow and blood micronucleus sampling at the end of a standard 28 day toxicity study. So we don't need to use any additional animals. We can incorporate it in to standard to testing. The same sort of recipee where you have 5 animals in 5 groups same sex per gorup. or one sex if theres no sex diffenrecens. Three dose levels if toxic. If non toxic then we might be a blue to get away with a single dose but if you intergrade it into a 28 day to study you would have three doses anyway. Recently the recommendatiosn for the number of cells to be scored have doubled again to avoid zeros.So we now score 4000 i8mature erythrocyte, polychromatic erythrocyte per animal in bone marrow and we use the ration of immature to mature erythrocyte to give us a measure of toxicity. Again we should measure

chemical in plasma so we can demonstrate exposure. But we can as we say measure micronuclei in the blood. And for this we can use flow cytometry and for that reason we can score a lot more cells. Now these young reticulocytes, we recomgnise them in a whole blood sample because they express e the CD71 marker, so we can have a flourecently tagged antibody to CD71 and idenitfy the youg reticulocytee population. Now the longer you treat the more likely it is that the spleen will remove an micro nucleated erythrocyte from the circulation. To compensatee for that we prefer to score larger numbers of cells thatn we would if we were doing it manual. In fact as long as we are picking up these young reticulocytes they are generally not recognised by the spleen so even if we have got micronuclei they tend not to be removed. But it is easy using flow cytometyr to sore anything from 20,000 to 2,000,000 cells per sample. So it realy gives us good stats power. And you don't need large blood sampels. So you can do cereal sampling you don't need to sacrifice your animals like you do to get at the bone marrow and you can add those cels on tot he end of a stadard tis study and your not interfering with the tissue that the pathologists want. So its really neat to be able to include this ito give a really good statistically power of information without using aditional animals and without interfering with the normal toxicology work. So flow cytomery of FACS is characterisation of single cells as they pass at high speeds through a laser. Light scattered forwards indicates refractive index and this is dependent on cell size, orgnaessl etc. So for example the nucleated cells in the blood will all scatter light forward so they can be gated out.So your not confusing the pooltion that you want to look at. You can gate out the nucleated cells because of the forward light scatter, light scattering side ways indicates density or cell surface granularity. So we can flow the blood sampels through the flow cytometer and look at the forward and the side ways light scattering and using our serious markers then we can pick up whats happening. This is just a trial when davids lab got invovled in this lightron were the experts located in rochester new york state and david did some experiments where we treated animals we took the blood. We fixed in this -80 freezers as these blood samples have to be kept very cold which is critical. Because if you don't what happens is is that CD71 cell surface marker becomes internalised os instead of sticking out from the cell surface me brain where we can identify it with an antibody, it terns inwards and so you cant identify those cells. So this -80 and methanol is absoullty critcal for good samples and we just split the samples we need half of them in davids lab and half over to cytron and they analysed them and that helped eatablesh the method. So you need a labelling solution bucks you need to pick up these CD71 markers ideatefy the politen that your interested in so you take the fixed blood sample add the labelling solution, incubate, add promidium iodide as a counter stain and run it through the flow cytometer. So we have already gated outthe nucelated cells. WE have sorted out the cells that are going to be within the gating properties that we are looking at are mature eyrthrocytes which won't have CD71. We have reticulocytes that will have CD71. Micro nucleated reticulocytes that will have D71 and will stain with prmidium iodide and then platelets which actually



come up yellow because of the stain tecniques that were using. so as we pass the blood through the flow cytometer. We get a dingla for a reticulocyte which dosnet contain a micronucleus and then we can get a single for a reticulaocyte so that will give us both FITC and PI. The platelet will only be pickedd up her ebecuase its yellow and so we can get a diatribetion





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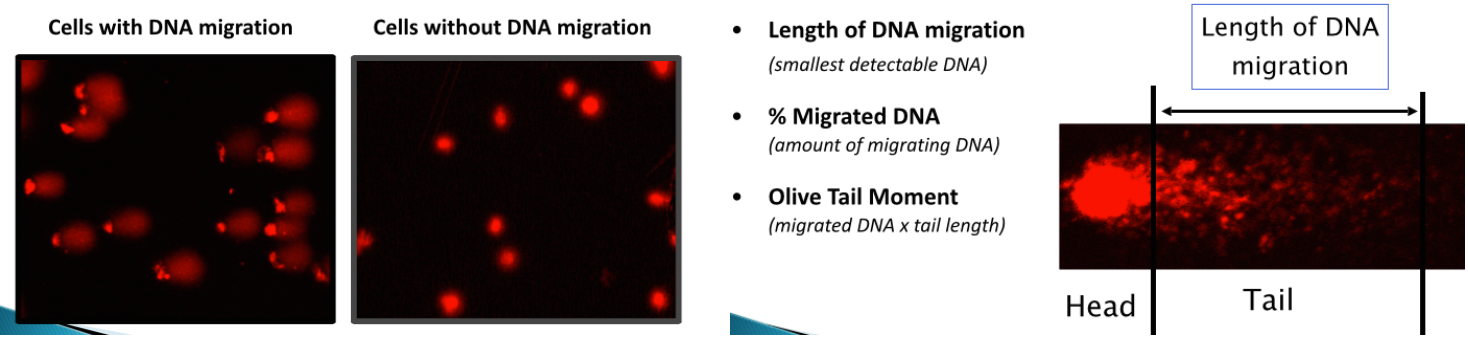
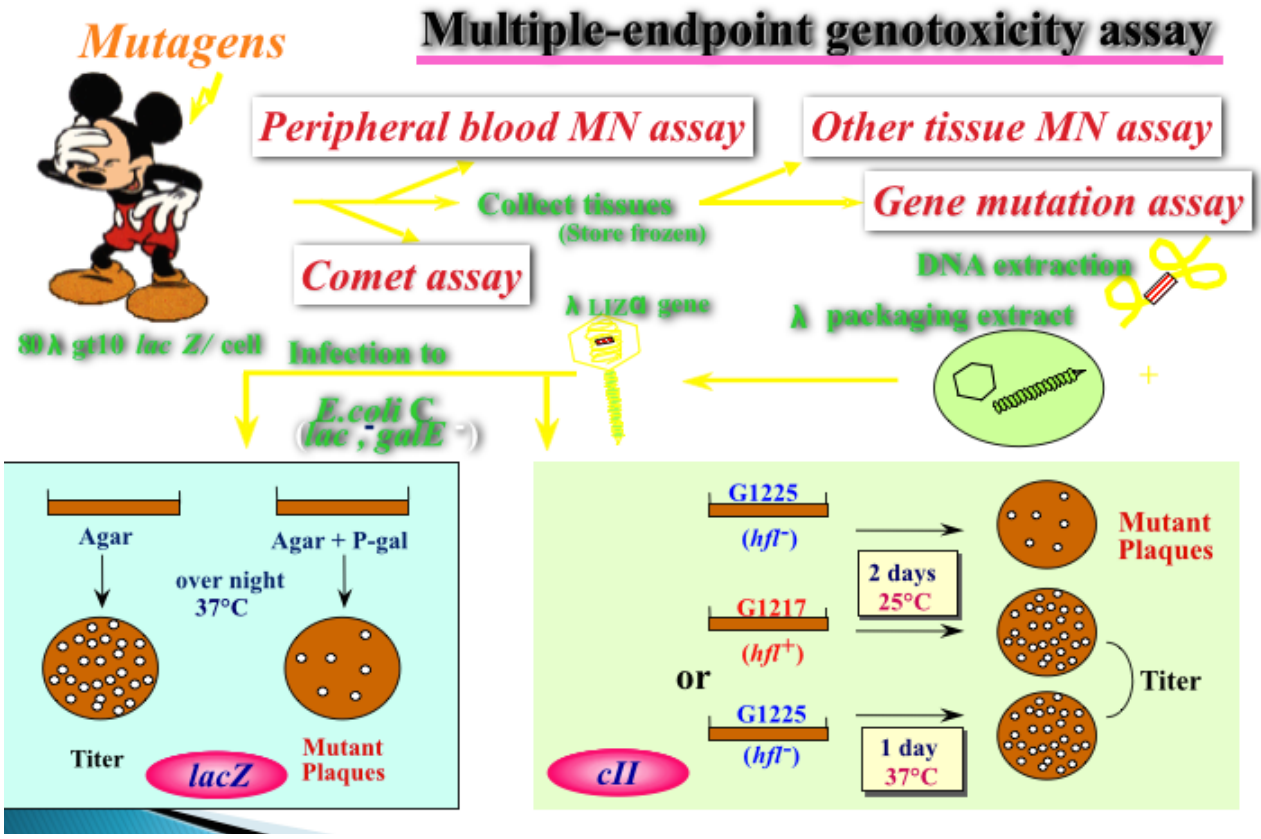
like the one sen below. Based not he florecent reactions that we are getting as the sample goes through the flow cytometer.So this is normal reticulocytes,. This is normal mature erythrocytes micronuleate reticuleytes and micr nucelated mature erythrocytes and so we can establish frequenciess and quantitate data.So this is for methlyethylsufaphate. Which we would normally have much fewer micronuculated reticulocytes in this box for control sampel. We can do micronuclei in control tissues other than bone marrow and blood, there is now a lot of data on measuring micronclei in the liver. Becuase you need dividing cells the old method used to be surgical intervention. We used to cut part of the liver away because it regenrates rapidly we could treat the animal whilst the liver is regenerating, but that surgiclal intervantion is demanding, so a group in japan started looking at youg rats. less than six weeks old were the liver is still dividing because it has not fully grown. and that looekd promising but then there were questions regarding the metabolism in a young rat is not the same as the metabolism in a mature rat. Maybe we could be geting misleading results. Recently a trial was done in japan where they dosed animals, they dosed teats for 14 or 28 days without any surgical intervention or without any additional checmiclas and ther is enough cell division within th eliver doorng that 14-28 day periodd that you can effectively measure micronuceli. We are just compiling this into a special year of mutation research which is going to be published soon. So loos as this is going to be very promising and we will probably end up with an OECD guideline for this in a few years. They also started looking at micronuclei in stomach and colon and again after 14 or 28 days there eis enough cell division going on that you can measure micronuclei in those tissues. so it looks as thouh we may be able to do site of contact micronucleus work as well. So liver as the major organ of emtaboism and stomach or colon as the major site of contact for the orally administered substances. looks as though as going to be an option for us going forward. So thats micronuclei. Ten minutes to finish of on strained barking.

So as we mentioned we need dividing cells for micronuclei and comabs os we cant easily do it in any old tissue. So whilst there is some promise that we can do micronuclei in liver and in the GI tract things like

kindness, spleen lung etc ar not eay. So we can take a step back and take a look at the kind of DNA strand brokerage that leads to chromosome aberrations and for this we tend to use the

commet assay , so this is a single cell gel electrophoresis assay where we are looking at the migration of broken DA in an electrical field. All you have to be able to do is creat a single cell suspension. So you can look at any tissue from which you can creat a viable single cell suspension. This si pretty easy for everything apart form skin. Skin is not easy to convert into a single cell suspension. It takes pretty aggressive digestion to brake down all the protective lawyers staring from the epidermis ETC to give yu single cells.ANd its difficult to get good viability form the skin but pretty much every other tissue you can make a single cell suspension and you can do the comment assay, and cells do not have to be dividing, so thats why its easy to do in a ny tissue. We tend to use alkaline conditions because this gives us a wider range of DNA lesions, and we no have last year and OECD guideline for the in vivo comment assay was adapted by OEC, people don't tend to do in vitor comment assay very much, We have got such good mammalian in vitro test any way that you don't really need to do it, so the emphaiss is really on in vivo use of the comment assay. So once we have got our cell suspension we mix it with the cell suspension with agrose, spread them on microscope slides. Gently lyse the cells so the DNA can be relased unwind the DNA so that any fragment can migrate. Carr out electrophoresis under standardised conditions then neutralise, stain and do image analysis which again is usually automated. Unwind ing at diffenret pHs expresses different types of damage and the more strand brakes we get the smaller the DNa framgetn, the more the migration. So if we were to do the test at neutral pH we would only really pick up double strand brakes and chromosome crosslinks. Not crosslinks because there difficult to detect because you have to have a high level of migration in you controles and you see a decrease in migratiuon in you treated cultures. So cross links should really have a big question mark against it. The standard test is not designed to detect cross linking agent but you can if you fiddle with the conditions. At alkaline pH we increase the number of genetic damages that we cnan pick up, so at 12 we pick up exactn repair sites and above 13 we also pick up alkalyl labile sites. So in order to get the best possible bang for your buck we tend to do the alkaline, at the gratertha pH 13 assay as standard. Why is it called the comment assay because the migration of the DNA on the gel looks like a celestial comet. There are different ways you can measure the extent of the damage, you can look at tail length or you can look at the amount or the percentage or DNA in the trial compared to the total or you do whats called a trial moment which is the amount of migrated DNA multiplied by the tail length. Most





people use the percent DNA in the tail so the percent of Dna in the trial, the percent of migrated DNA, that is the most widely used and widely recommended perimeter. Controle cells with very little DNA migration, cells treated with positives ccontrole chemicals have lots of migration. This was just for a bit of fun where ea college of david kurklands in japan did a few years ago said we dont need to do all of these in vivo test. all we need is one super animal and we can do anything. We can do micornuclei in blood, we can do micronuclei in other tissues, if we do this study in a transgenic animal then we can do mutation as well. So lets do everything in one animal. Ets have a super micky and we can do all of these end point in a single animal. Which may happen one day and we a re moving a little bit towards it but were not quite there yet. So again as divd mentionedd at the ned of the first talk all of this data generation is very intersting but there are still a lot of diffenret way in which people will cconsider wether the deathly have got in formunt of them is postive or not. Some prefer statistical analysis.Some prefer a fold increase over control. others look t the historical control range and other will perhaps use a preset level as we do fofr the mouse lymphoma assay. Dose response is important, if you do the experient more than once then reproduceability is important anw what we can see is that this is how it used to be and we have had several battles with OECD to try and get some better thiniking on this and in the latests guildnes to be approved we have come up with a multiporngedapporoch that people seem to buy into. And what we have said is ask three or four questions of your data set.

1) is R any point statistically significant.

2) do any of the point exceed the historical control range (and we have had to define very caerfully how a historical controle range would be stablished)
3) Is there is a dose repsonce
4) and if you did more than one experiment ar the results reproducible.

So its statistical significance, historical control range, dose response and reproducibility. you could substitute the pre set levels for historical control range if there is a good basis for it, and what we have said is, if you answer yes to all of those question and your data is statisitcally significant, if it exceed the historical control range or a pre set level, it has a does response and is reproducible then that is clearly positive. IF you answer no to all of these then it is clearly negative. Anything in between is going to be equivocal and it is going to need further investigation. You with do more experiments where you change the conditions, you increase the number of cells you score you increase the number of sampling times or something like that tor you move to different test system and look at different end poitns. So if you answer yes to some but no to others and it is neither clearly positivee nor negative, then do more work is basically what it comes down too. But at least we now have some broad conensis over thefts was to try to conclude on the data thats in front of us for lall these diffenrt tests and the common approache tat we can use for evey test system.It docent matter if its the ames test or a comment assay or a micronuclease test we can apply the same criteria to every test system and that at least gives us some consistency. so hopefully going forwards thereyll be more agreement over the way to interpret these tests.