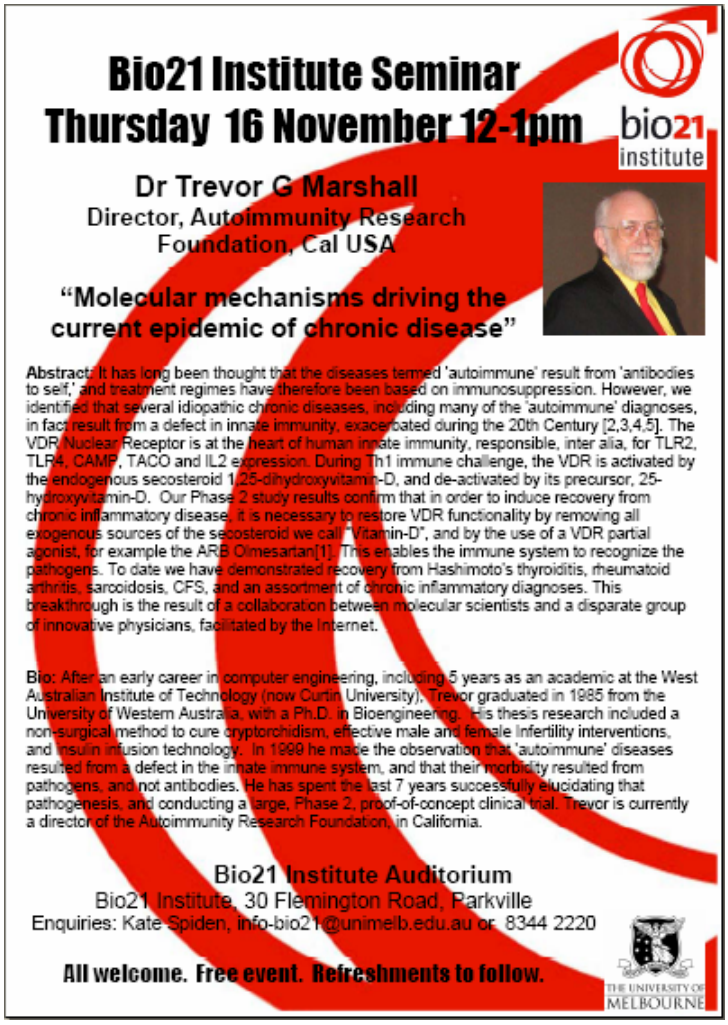
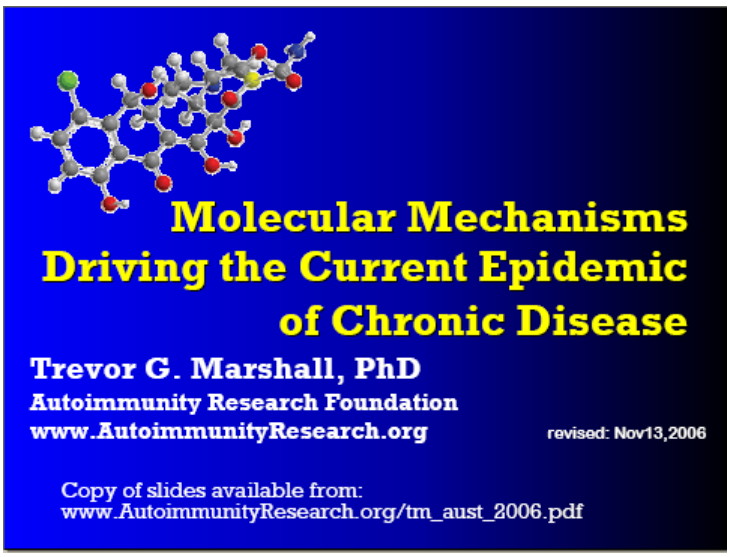
Bio21 Institute Seminar

UNIVERSITY OF MELBOURNE, THURSDAY 16 NOVEMBER 2006

Molecular Mechanisms Driving the Current Epidemic of Chronic Disease

Trevor G. Marshall, PhD
Director, Autoimmunity Research Foundation, Thousand Oaks, California
School of Biological Sciences and Biotechnology, Murdoch University, Western Australia
Patron, Australian Autoimmunity Foundation





This presentation is available on the DVD "The Science — Marshall Protocol" available upon request from the Autoimmunity Research Foundation (http://www.AutoimuunityResearch.org). A lower quality real-time streaming version may be found at Internet URL http://autoimmunityresearch.org/bio21a.ram .

TRANSCRIPT OF PRESENTATION

Introduction by Dr Pillip Batterham, Department of Genetics, University of Melbourne
0.07 / 1:23/02

DR. BATTERHAM: I'd like to welcome you to today's seminar. We pride ourselves in BIO21 in being very multidisciplinary and what could be more multidisciplinary than a geneticist standing up to introduce someone trained in electrical engineering talking about autoimmune disease.

Trevor Marshall graduated electrical engineering from the University of Adelaide. He taught at the University of Technology in in New Guinea, and Curtin University in WA and earned a master's degree in engineering from the University of Adelaide in 1978. And undertook Phd studies in Charles Gardiner hospital in WA, the Hospital for Sick Children in Toronto. He earned a PhD for mathematical modeling of insulin-glucose homeostasis in diabetic and healthy individuals in 1985 from the University of WA.

To make a long story short, in 2004 Dr. Marshall established the Autoimmunity Research Foundation based in California. He's currently visiting Australia, he is currently visiting Australia to support the launching of the Australian Autoimmunity Foundation and to locate partners for Phase 3 clinical trials for drugs treating autoimmune conditions.

I'd very much like to welcome you today to the seminar and after which there will be a light lunch for you in the foyer where you will be able to join Dr. Marshall. Thank you.

Title Slide
1:42 / 1:12/17

DR. MARSHALL: Thank you very much and thank you for giving me the opportunity to visit Melbourne again at this lovely institute and wonderful auditorium.

Well, I'm going to talk about Molecular mechanisms which I believe are driving the current epidemic of chronic disease.

As you can see at the bottom of the screen there's a URL there where you can download a copy of these slides from the web and have a closer look at them at your leisure. That's echoed on the final slides so you don't have to write too quickly.

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Published Sources - citations

This presentation will draw upon the work of others, usually referenced by a PubMed ID number 'PMID:', and upon my own recent peer-reviewed publications. These include:

Marshall TG: VDR Nuclear Receptor Competence is the Key to Recovery from Chronic Inflammatory and Autoimmune Disease. 'Days of Molecular Medicine', 2006. http://autoimmunityresearch.org/karolinska-handout.pdf

Marshall TG: Molecular genomics offers new insight into the exact mechanism of action of common drugs - ARBs, Statins, and Corticosteroids. FDA CDER Visiting Professor presentation, FDA Biosciences Library, Accession QH447.M27 2006

Marshall TG, Lee RE, Marshall FE: Common angiotensin receptor blockers may directly modulate the immune system via VDR, PPAR and CCR2b. Theor Biol Med Model. 2006 Jan 10;3(1):1. PMID:16403216

Waterhouse JC, Marshall TG, Fenter B, Mangin M, Blaney G: High levels of active 1,25-dihydroxyvitamin D despite low levels of the 25-hydroxyvitamin D precursor - Implications of dysregulated vitamin D for diagnosis and treatment of Chronic Disease. In Vitamin D: New Research. Volume 1. Edited by: Stoltz VD. New York: Nova Science Publishers; 2006. ISBN: 1-60021-000-7

Published Sources – citations - continued

Marshall TG, Fenter BJ, Marshall FE: Antibacterial Therapy Induces Remission in Sarcoidosis (in English). JOIMR 2005;3(1):2 Available from URL http://www.joimr.org/phorum/read.php?f=2&i=107&t=107

Marshall TG, Fenter B, Marshall FE: Antibacterial Therapy Induces Remission in Sarcoidosis. Herald MKDTS 2004g; Volume III: Release 1. (The Journal of the Interregional Clinical-Diagnostic Center, Kazan, Invited paper, Special issue on Sarcoidosis. Published in Russian translation). ISSN: 1726-6149

Marshall TG, Marshall FE: Sarcoidosis succumbs to antibiotics - implications for autoimmune disease. Autoimmunity Reviews,2004; 3(4):295-3001 http://dx.doi.org/10.1016/j.autrev.2003.10.001 PMID: 15246025

Marshall TG, Marshall FE: Antibiotics in Sarcoidosis - Reflections on the First Year. JOIMR 2003;1(3):2

Marshall TG, Mekhiel N, Jackman WS, Perlman K, Albisser AM: New microprocessor-based insulin controller. IEEE Trans Biomed Eng. 1983 Nov;30(11):689-95. PMID: 6662523

Keogh EJ, MacKellar A, Mallal SA, Dunn AG, McColm SC, Somerville CP, Glatthaar C, Marshall T, Attikiouzel J: Treatment of cryptorchidism with pulsatile luteinizing hormone-releasing hormone (LH-RH). J Pediatr Surg. 1983 Jun;18(3):282-3. PMID: 6135766

Full list of publications at http://TrevorMarshall.com/papers.htm

Disclosures, FDA applications in process

Many of the disease states described in this presentation, including neurological states, are not generally accepted as being caused by pathogens. Much of this presentation is based upon leading edge science, not on "weight of evidence." A Phase-2 clinical trial is ongoing.

Even when shown the science, and the microscopy, some experts still disagree with the existence of persistent pathogens. The FDA has now designated long-term use of Minocycline and Clindamycin in the treatment of Sarcoidosis, but not in the other indications I discussed.

Although FDA applications are current for PTLDS and Sarcoidosis, marketing approval has not yet occurred, and use of these principles is therefore 'off-label'

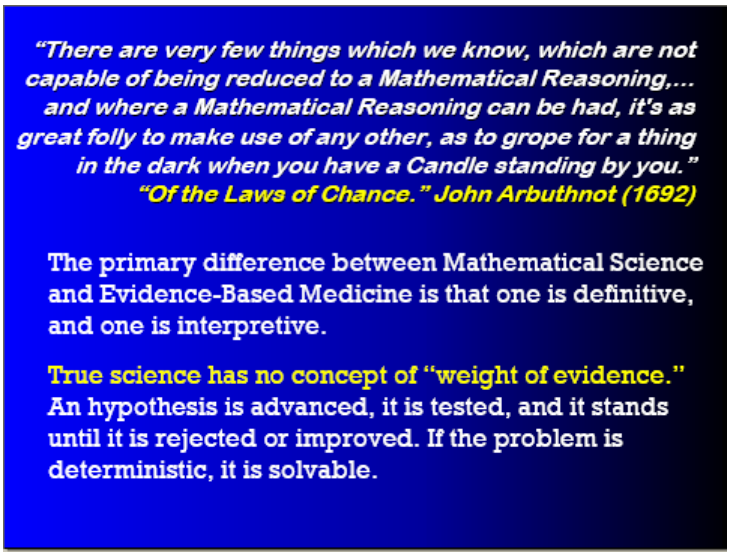
The following FDA OOPD applications are currently active: 05-2131, 05-2133, 05-2134, 06-2287,06-2288,06-2289, and their text should be consulted for further information

I've put some citations and disclosures and things on there. We have a number of applications current with the Food and Drug Administration in the USA.

We've had [phase 3 testing designation] approvals for two antibiotics, clindamycin and minocycline in sarcoidosis. We're also talking with them about chronic fatigue syndrome and a number of other studies that we're trying to get underway to move as quickly as possible and consolidate on the Phase 2 studies that we ourselves did.

And there's a list of the FDA application numbers at the bottom and any of you can look up those numbers and look at it in more detail at the science that I'm going to cover in the presentation.

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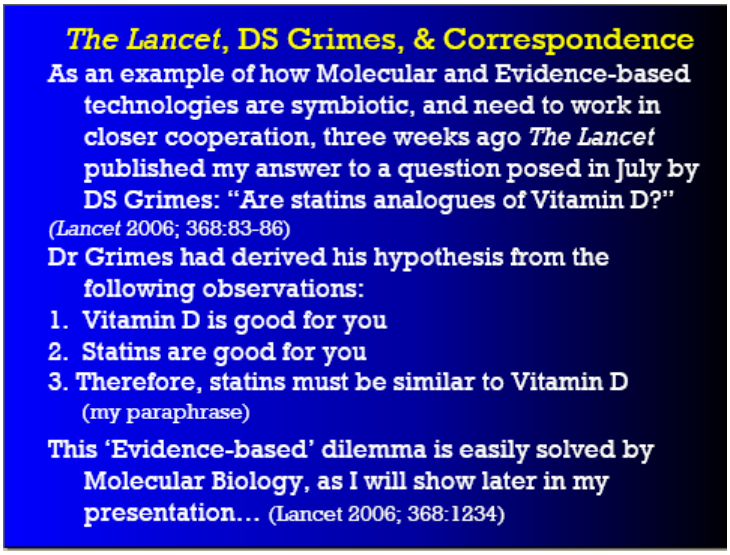
"Of the Laws of Chance"
3:02 / 1:12/17

I usually start with a quote from John Arbuthnot, 1692. John Abiernot wrote the first book on statistics of the Laws of Chance and of course it was about cards, playing cards, but it was the first formal text on statistics. But he wrote this wonder passage that he says: There are very few things which we know which are not capable of being reduced to a mathematical reasoning. And where a mathematical reasoning can be had, it's as great a folly to make use of any other, as to grope for a thing in the dark when you have a candle standing by you.

The primary difference between mathematical science and evidence-based medicine is that one is definitive and one is interpretive. Mathematical science, biomedical science tries to define relationships between entities. And evidence-based medicine tries to place reliable interpretations on observational data. Primarily, I mean, there's obviously some fluidity there.

True science, the primary difference between them is that true science has really no concept of weight of evidence, that the hypothesis is advanced. E equals MC squared, for example, it's tested and it stands until it's improved or rejected. And if the problem is deterministic, it's solvable.

Now of course in medicine most of the problems are not deterministic — yet, but as our knowledge advances more and more of the medical mysteries, if you like, clinical mysteries, start to succumb to molecular biology and that's what I'm going to talk about today.



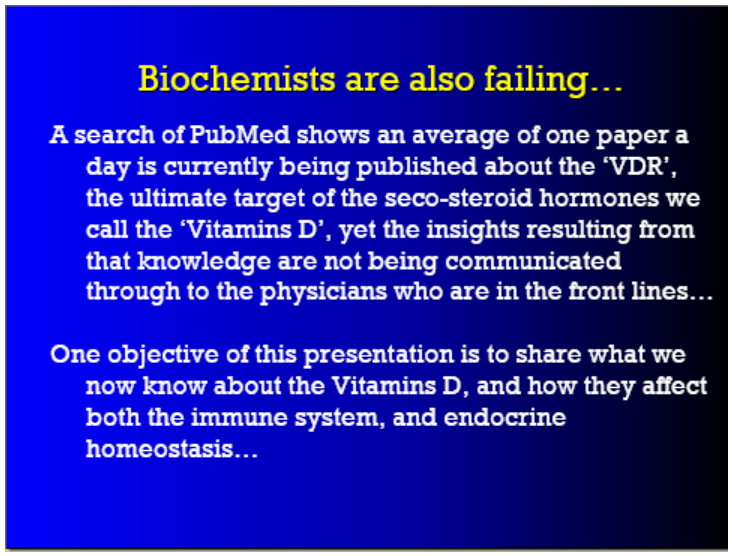
The Lancet, DS Grimes, & Correspondence
4:52 / 1:12/17

It's very important that both the molecular biology and the clinical sides of medicine work together in close concert. And this is the example that I'm going to discuss. It's from the Lancet. Dr. D.S. Grimes wrote a hypothesis in July of this year [2006] which was titled: "Are Statins Analogues of Vitamin D?" And Dr. Grimes had derived his hypothesis from the following observations: Vitamin D is good for you, statins are good for you, therefore, statins must be similiar to Vitamin D. Now I'm being very harsh there and it's a much better hypothesis than that. You need to look in detail at it, but that's the essence that I pulled out of it.

The problem that Dr. Grimes has in analyzing this particular question using evidence-based medicine is that there are far too many degrees of freedom. You have the degrees of freedom related as to how the statins act — and clinical medicine has no idea how the statins act, how Vitamin D acts — and clinical medicine has very little idea of how Vitamin D acts. And then of course you've got the experimental degrees of freedom as well that are going to come into the data and make it very difficult to draw a good conclusion. And of course Dr. Grimes really wasn't able to do so.

However, my letter was published which did solve the dilemma because, by recourse, the molecular biology, I do know what the statins do and I've also got a pretty good idea what the Vitamine D does. And because I can reduce that to mathematics with the molecular biology it's very easily communicable to people that can understand molecular biology. And later in the presentation I'll go over the statins.

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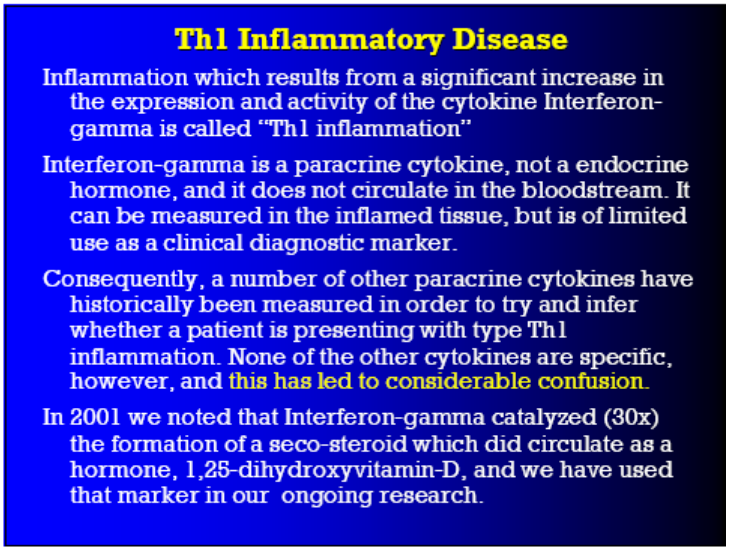
Biochemists are also failing...
6:44 / 1:12/17

So clearly that's a gap in evidence-based medicine, but you know the biochemists are also failing because a search at PubMed shows an average of one paper a day is currently being published about the VDR, the Vitamin D receptor. And if Dr. Grimes had been following this literature it would have helped him alot in understanding at least half of his conceptual problems.

Yet, the insight resulting from the knowledge about the nuclear receptor, the VDR, are not being communicated through to the physicians who are on the front lines.

One-paper-a-day, this tremendous weight of evidence. Somebody the other day wrote that I am alone in saying that Vitamin D is a steroid. Well, I'm sorry. You cannot read these one-paper-a-day on the VDR and the nuclear receptors in PubMed without understanding that everybody that's working in this field has come to that same conclusion.

But the biochemists, the molecular biologists, are not getting that information across to the clinicians. It's very important that both phases of medicine work in concert. And one objective of this presentation is to share what we now know about the Vitamins D and how they affect both the immune system and the endocrine homeostasis.

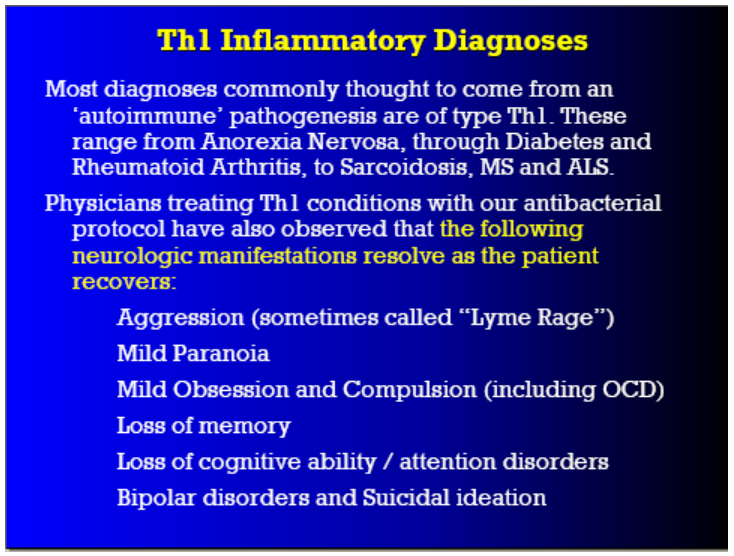


Th1 Inflammatory Disease
8:03 / 1:12/17

Th1 inflammatory disease is defined as disease which results from a significant increase in the expression activity of interferon gamma. It's a paracine cytokine, very hard to measure and it doesn't circulate in the bloodstream, therefore, most clinicians tend to look at other cytokines and try to draw an inference about what interferon gamma probably is.

Well, unfortunately that doesn't work very well and it's led to considerable confusion in trying to determine whether patients are exhibiting inflammation of type Th1 or Th2. Th1 being innate immunity. Th2 being adaptive immunity, broadly speaking.

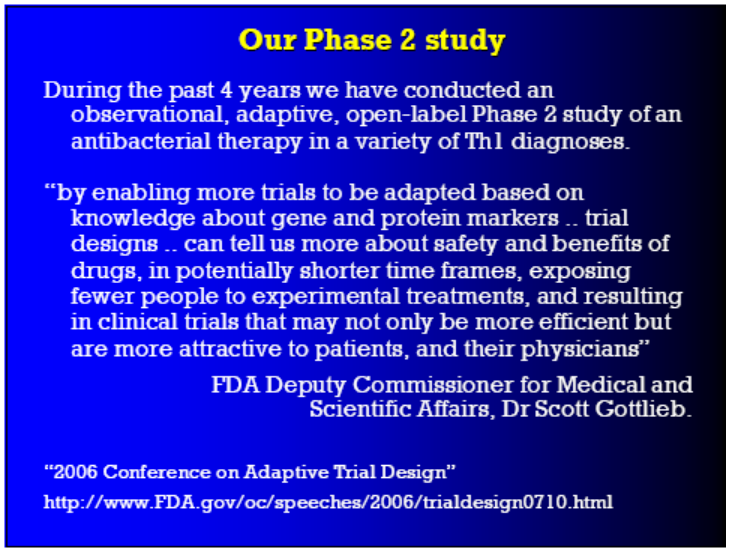
Now in 2001, we noted that interferon gamma catalyzed by a large amount thirty times, up to thirty times the formation of a secosteroid which did circulate as a hormone: 1,25 dihydroxyvitamin D. And we've used that marker in our ongoing research.



Th1 Inflammatory Diagnoses
9:03 / 1:12/17

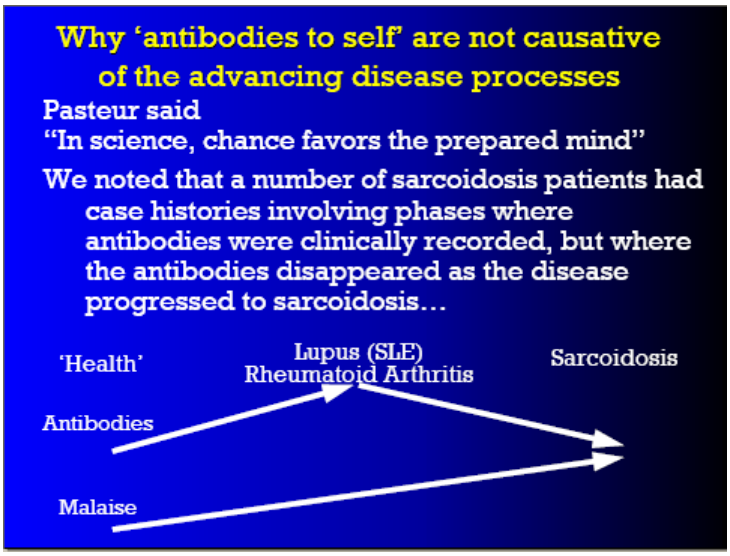
Most diagnoses are normally thought to come from an autoimmune pathogenesis or of type Th1. These range from anorexia nervosa through diabetes and rheumatoid arthritis to sarcoidosis, MS, and ALS. But physicians treating these Th1 conditions with our antibacterial protocol in the Phase 2 trials have also observed that the following neurologic manifestations resolve as the patient recovers: The inflammation disappears, excessive aggression starts to disappear, mild paranoia, mild obsession, and compulsion — we actually have classic obsession compulsion disorder amongst our cohort, loss of memory, loss of cognitive ability, and attention disorders, bipolar disorders, and even suicidal ideation.

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Our Phase 2 study
10:02 / 1:12/17

So over the last five years from early 2002, we have conducted an observational, adaptive, open-label Phase 2 study of an antibacterial therapy in a variety of Th1 diagnoses. I've given a quote there from the FDA Deputy Commissioner for Medical and Scientific Affairs. It's a good quote and the paper, the complete paper at the bottom I recommend to any of you who are involved in clinical study design. This represents a major change in the thinking of the regulators about how trials should be designed. They should be designed to be successful and actually produce results is what the FDA is starting to say now. I'll just refer you to his paper and move onto our next slide.



Why 'antibodies to self' are not causative of the advancing disease processes
10:46 / 1:12/17

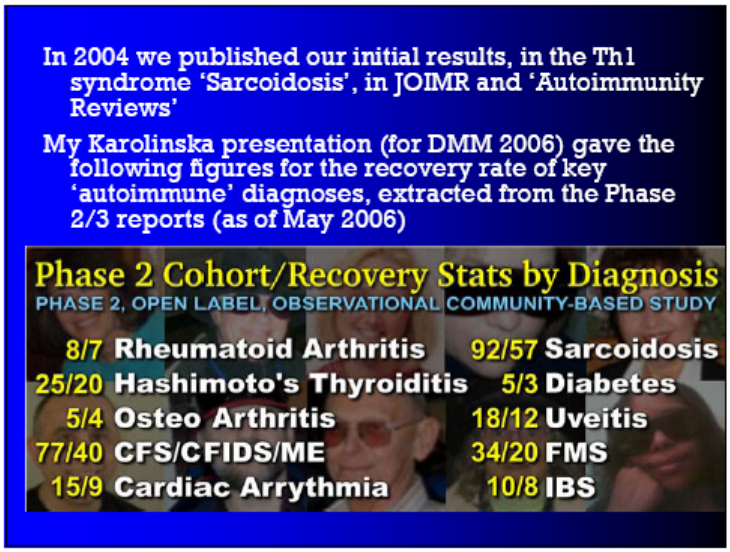
One of the first big breakthroughs we came across was to understand that antibodies themselves were not capable of making the patients as sick as they were presenting.

Now Pasteur said that "in science chance favors the prepared mind." So there's two elements for success in science: Sheer luck — chance, and when it's in front of you, you can see it and understand what it is, that's the prepared mind. And you need both those elements to make discoveries. And we were lucky. We noted, we had started with the disease sarcoidosis which is really an end-stage Th1 disease similar to MS and ALS. It kills pretty quickly after diagnosis. And we noted that the number of sarcoidosis patients had case histories involving phases where antibodies had been clinically recorded, but where the antibodies have disappeared as the disease progressed to sarcoidosis.

So I've shown time one here health and time 'n' here, sarcoidosis, and in the middle we had, and I'm specifically looking at lupus SLE and rheumatoid arthritis diagnoses, both of which involve specific antibody presentations in order for the diagnosis to have been made, and the patients later progressed from those diseases to the end-state of sarcoidosis.

The interesting thing was that sarcoidosis there are usually no antibodies. There is usually a very low sedimentation rate indicating that there's no real obvious pathogens, the immune system is not feeling anything. And that's one of the reasons it's such an idiopathic and has been such a puzzling disease.

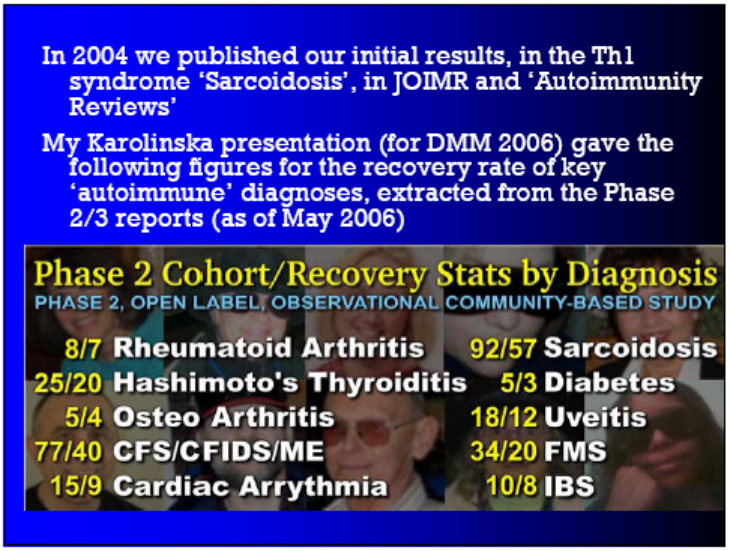
Yet, when you look at the malaise suffered by the patients, the malaise is a straight line. There is no peaking of the malaise that was coincident with the peaking of the antibodies. In fact, the antibodies just seem to be bystanders, if you like, of the main disease process. When we understood that, that was about in late 2003 – early 2004, we opened up the Phase 2 study to allow different inflammatory diagnoses to become part of the study. And in particular that was when we started taking in the chronic fatigue syndrome patients, the lyme disease, chronic lyme disease patients, the rheumatoid arthritis, Hashomoto thyroiditis, all the rest of them.

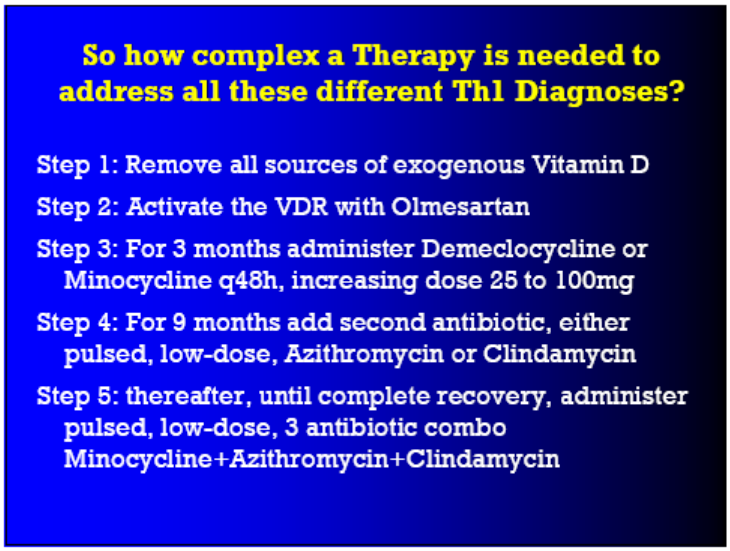


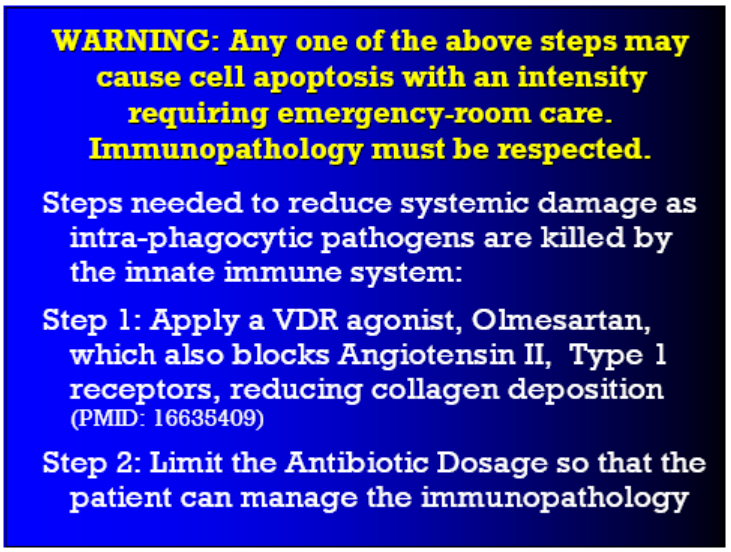
Phase 2 Cohort/Recovery Stats by Diagnosis
13:25 / 1:12/17

And this is what we found. In 2004, we published our initial results which were basically just of the sarcoidosis subset of the

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cohort in *Autoimmunity Reviews*. But I travelled to Karolinska Institute in Stockholm in May [2006] and I gave the following figures for the recovery rate of key autoimmune diagnoses which were extracted from our Phase 2/3 reports as of May 2006.

Now these are people who have been on the protocol typically eighteen months and are well advanced on their road to recovery, and I have two numbers here, for example rheumatoid arthritis, we have the total number in the cohort, that have been in the cohort eighteen months which is relatively low at that point, eight. And then the number that we're reporting recovery in this case it's very high. It is very high in this disease which is seven. Hashimoto is twenty out of twenty-five. Osteoarthritis we're at a five. CFS, we've been enrolling for quite a long time so we have a large number in the cohort. And forty out of seventy-seven were reporting recovery there after eighteen months. Cardiac arrythmia. Sarcoidosis has the largest numbers of all because that's how we started the study rolling. The diabetes, three out of five. Uveitis, twelve out of eighteen. Fibromyglia syndrome twenty out of thirty-four. And irritable bowel syndrome, eight out of ten. And that's just the subset of the major diagnoses of the subjects in the cohort.

So how complex a Therapy is needed to address alll these different Th1 Diagnoses?
15:00 / 1:12/17

So how complex a therapy was needed to address all of these different Th1 diagnoses and induce a response from these disparate, apparently disparate, groups of patients?

• Step one was to remove all sources of exogenous Vitamin D.

• Step two was to activate the VDR nuclear receptor with Olmesartan. Olmesartan is a relatively new sartin drug. It's a very active ligand, very polar ligand, and it targets a number of receptors. And I'll talk more about that as we move on.

• Now for the first three months we administer a tetracycline, preferably demeclocycline or minocycline every 48 hours starting at an extremely low dose of 25mg every 48 hours working up to a 100mg over the three months, typically.

• Then for the next nine months we add a second antibiotic, either a low dose of azithromycin or clindamycin, whatever the patients can handle. And the patients at this stage of the therapy after the first three months have great deal of difficulty tolerating one-eighth of the tablet of azithromycin every ten days added to the antibiotic cocktail that they're taking, which at that point is minocycline. By taking out the exogenuous Vitamin D and activating the innate immunity via VDR, the sensitivity of the antibiotics is increased many, many times over.

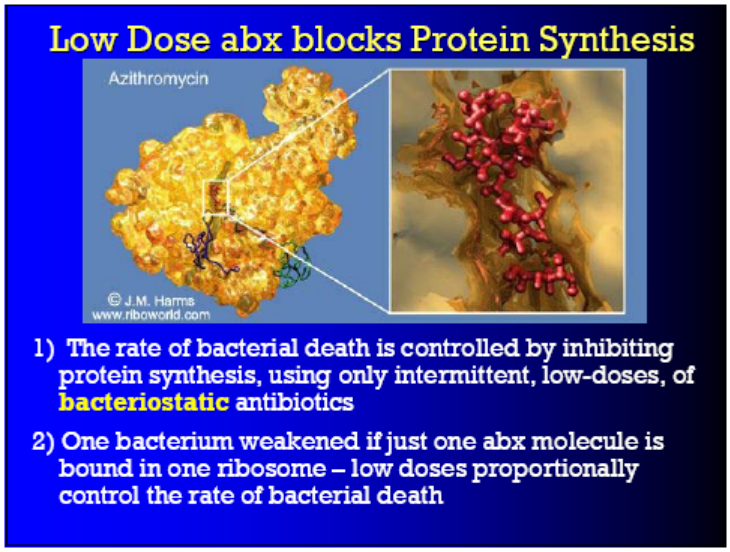
• And thereafter until complete recovery we administer pulsed, low dose, three-antibiotic combo: minocycline, azithromycin, and clindamycin.

WARNING
16:42 / 1:12/17

And this is the warning which I was told to put in by my FDA liaison guy: **Any one of the above steps may cause cell apoptosis with an intensity requiring emergency room care. Immunopathology must be respected.**

Immunopathology is the lastest keyword for the damage done to the body by the immune system doing its job.

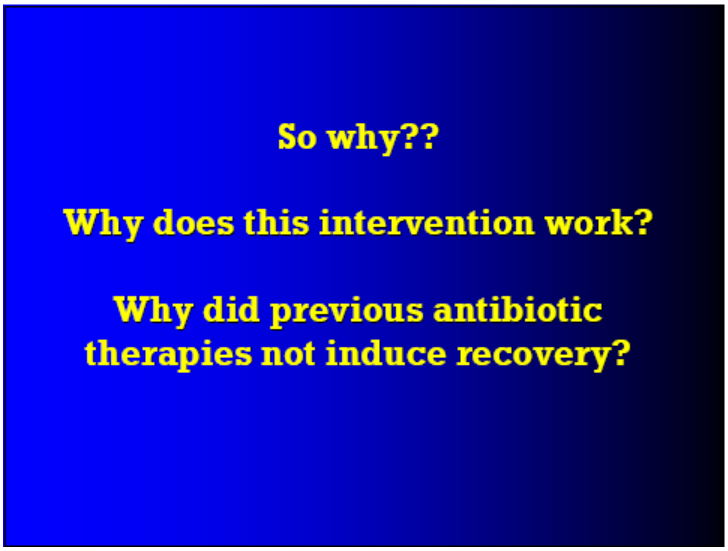
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So the immune system kills the bacteria and in this case you'll see the bacteria intracellular. You lose cells, the cells die when the bacteria are killed inside them. The cytokine storm and just cleaning out the dead cells profoundly affects the malaise and general health of the patient. So steps are needed to reduce systemic damage as the intraphacytic pathogens are killed by the innate immune system. And I've got the steps there and you can look them up from the PDF on the web.

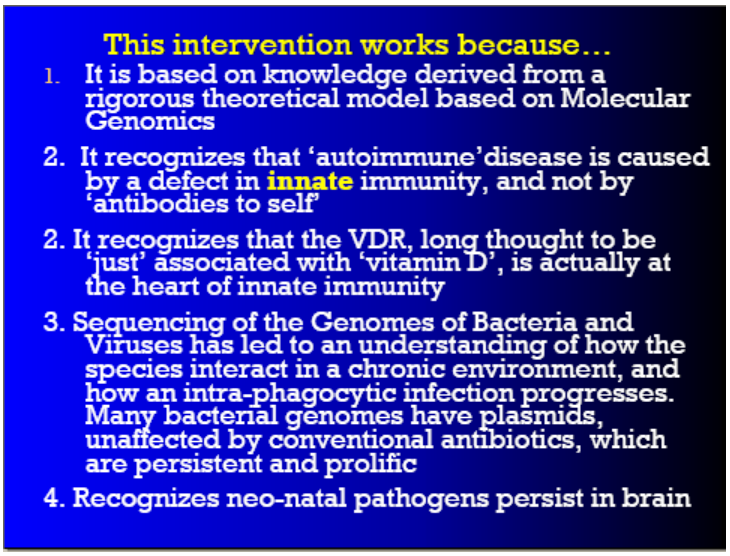
Low Dose abx blocks Protein Sythesis
17:45 / 1:12/17

The key thing is that the rate of bacterial death is controlled by inhibiting protein synthesis in the pathogens using intermittent low doses of bacteriostatic antibiotics. And particularly bacteriostatic antibiotics that target the translation of mRNA into proteins, target the ribosome, the 70S bacterial ribosome. One bacterium is weakened it's just one abx molecule is bound into one bacterial ribosome. Low doses proportinately control the rates of bacterial death.



So why? Why does this intervention work?
18:24 / 1:12/17

So why? Why does this intervention appear to work when previous antibiotic therapies didn't induce recovery?



The intervention works because...
18:32 / 1:12/17

The intervention works because, one, it's based on knowledge derived from a rigorous theoretical model which is based on molecular genomics.

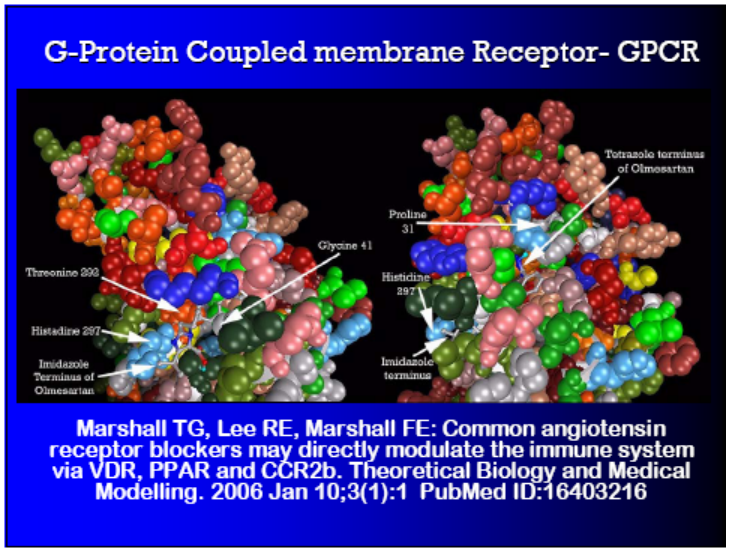
The intervention recognizes that autoimmune disease is caused by a defect in innate immunity and not by antibodies themselves.

It works because it recognizes that the VDR, long thought to be just associated with Vitamin D, is actually at the heart of innate immunity.

And it works because sequencing the genomes of the bacteria and viruses has led us to an understanding of how these species interact in a chronic environment — how they interact building up slowly in a person's body during the course of a lifetime, and how the horizontal transfer of DNA occurs between the species, blurring the distinction between species and allowing one species to acquire the ability to invade the immune system from another species.

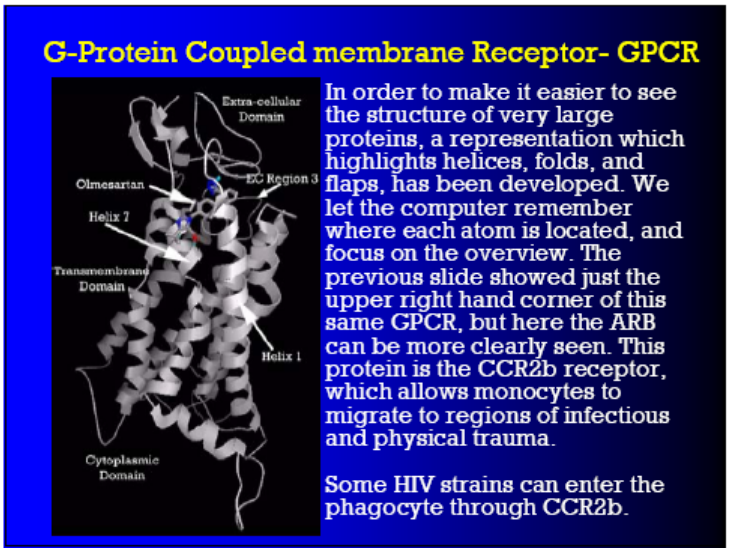
And finally we recognize that the neonatal pathogens can persist in the brain.

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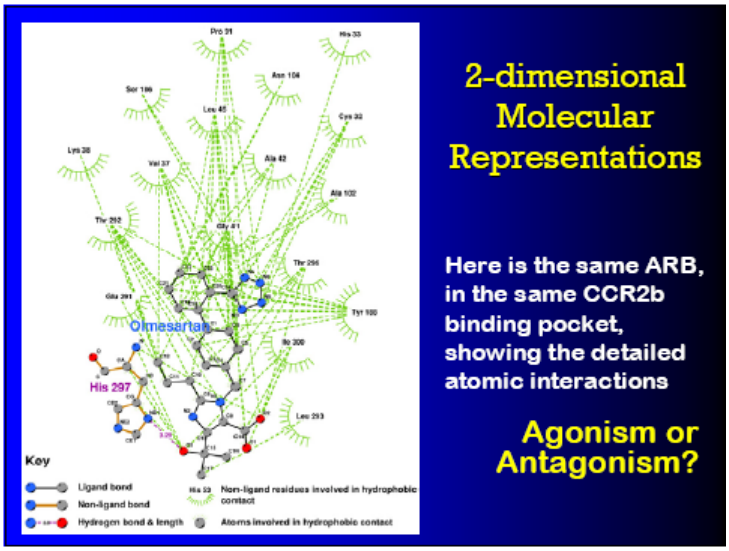
G-Protein Coupled membrane Receptor-GPCR
19:50 / 1:12/17

So let's go through those points one at a time. I just got a picture there of one way of visualizing a large protein. In this case a G-Protein coupled receptor GPCR. And we've got different atoms there with force field radiuses and they were pretty colors and they look nice and stuff but it's really hard to analyze much from an image like this.



G-Protein Coupled membrane Receptor-GPCR
20:10 / 1:12/17

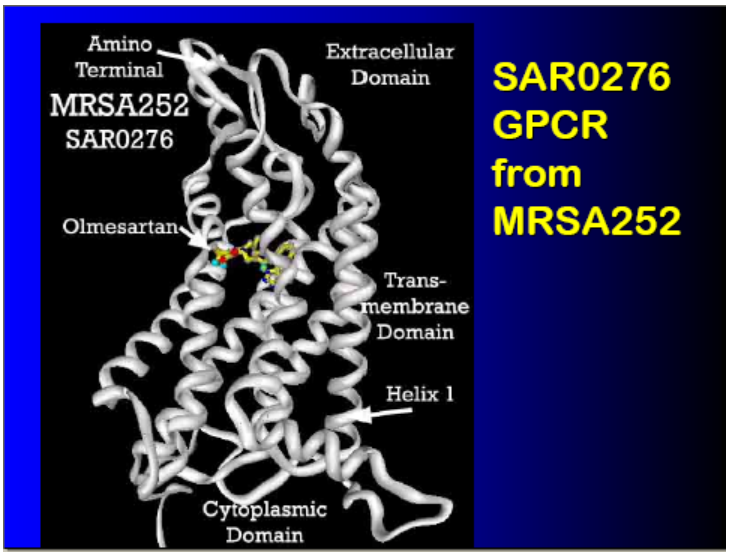
So biochemists tend to prefer to draw the large proteins in terms of helical structures, loops, and the other structures that are associated with the larger proteins, and the folds are visible. There is more detail in that on the PDF.



2-dimensional Molecular Representations
20:57 / 1:12/17

When you want to look at interatomic reactions to try and figure out whether you've got agonists or antagonists, then you have to sit down a little bit more carefully and these 2-D plots, I prefer you use the program called Ligplot, L-i-g-p-l-o-t, which produces this type of diagram. Makes it extremely easy to see how any of the amino residues such as this one exactly interacts with the ligand. Each particular force is a significant, interatomic force is significant is plotted on a graph like this.

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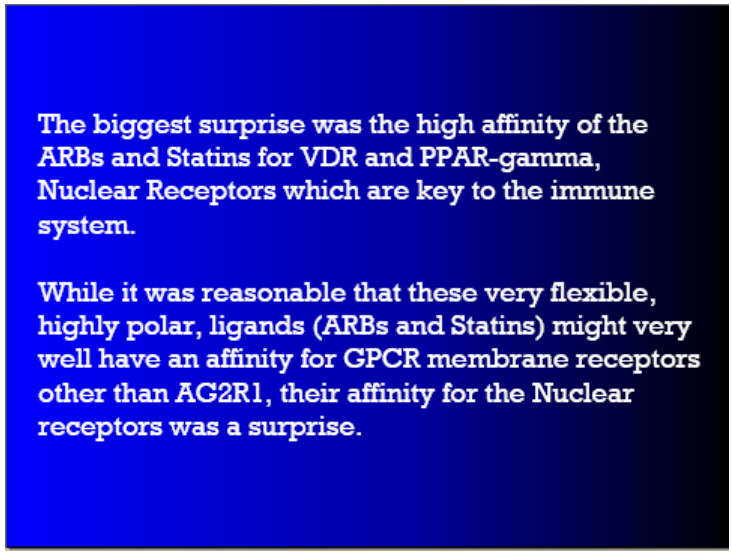
SAR0276 GPCR from MRSA252
21:08 / 1:12/17

So what can you do with all this technology?

This is a protein, SAR0276 which was taken from the genome of a staph bug called MRSA252. And as you know MRSA252 is one of the super bugs. This is a particularly nasty beast indeed. But its genome is being fully sequenced by the Sanger Institute and is one of the 363 fully sequenced bacterial pathogens that's now available in the gene bank.

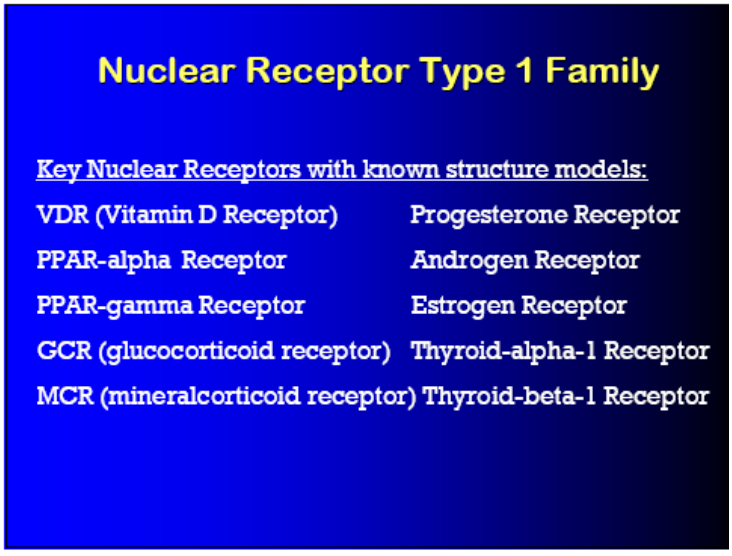
And when you go to the DNA and start looking for proteins within that genome which are likely to have some pathogenic intent, this is the first one that I came up with. I went looking for a GPCR that was similar to CCR2B and CCR4, CXCR4. For example, CXCR4 is where HIV enters the phagocytes through those receptors. And I was looking for some way that these organisms facilitate egress and ingress to and from the phagocytes.

Here is what I found, but what's particularly interesting is that I've got this Olmesartan, this little ligand, the angiotensin receptor blocker in here, and it binds very, very firmly into an active or an inactiving position in that GPCR. It's a very highly polar ligand and bascially any GPCR you find in a body, these new sartans and statins will have some affinity for. Olmesartan happens to have a high affinity for this particular bacterial protein, GPCR.



The biggest surprise...
22:58 / 1:12/17

But you know what really surprised us was that the ARBs and statins also had a very high affinity for the nuclear receptors, particularly for VDR, PPAR gamma, and the other nuclear receptors which are key to the immune system. So we were surprised because while it was reasonable that these very active ligands would bind into transmembrane receptors — the GPCR is a transmembrane receptors — for them to bind into nuclear receptors and into the ligand binding pocket in agonistic and antagonistic locations was total surprise.

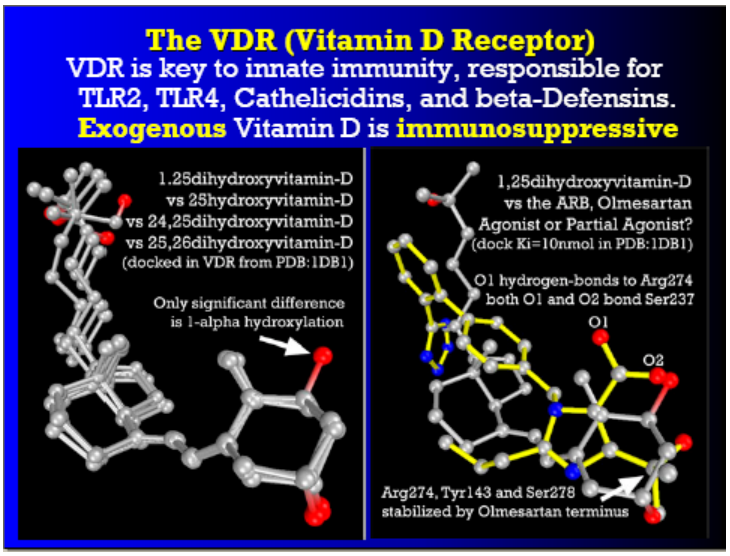


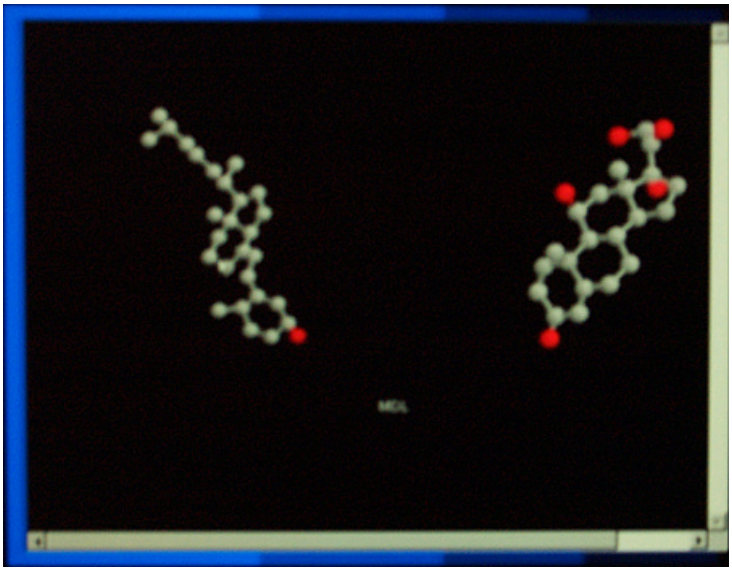
Nuclear Receptor Type 1 Family
23:38 / 1:12/17

The nuclear receptive family I have on this slide I'm sure most of you would be familiar with what we have there: The type 1 nuclear receptors are the VDR — the Vitamin D receptor, the PPAR alpha receptor, the gamma, the glucocorticoid receptor — that's the home for cortisol, mineralcorticoid recceptor, progesterone receptor, androgen receptor, estrogen receptor, thyroid alpha-1, thyroid beta-1.

Now we have good x-ray structures of all of these nuclear receptors so they're quite easy to study how drugs actually affect these nuclear receptors because we know what the nuclear receptors look like, very precisely.

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The VDR (Vitamin D Receptor)
24:17 / 1:12/17

So when we look at the Vitamin D Receptor, at this point each one of those one-paper-a-day that's coming out on the VDR is increasing our knowledge on what the VDR does.

But the key things that I want to point out at this point is that VDR is the key to innate immunity. It's responsible for the expression of toll-like receptor 2, toll-like receptor 4, the cathelicidin anti-microbial peptides and the beta-Defensins.

The beta-Defensins are key to the way that the gut, the immune system in the gut, handles flora and handles pathogens which appear in the gut. Beta-Defensins and alpha-Defensins are very active there.

The cathelicidin anti-microbial peptides typically tend to be more active within the phagocytes themselves.

TLR4 is the receptor that is sensitive to lipopolysaccharide, the prototypical bacterial marker. TLR4 is sensitive to lipopolysaccharides. TLR2 is sensitive to other bacterial lipo proteins.

So if you're not expressing the TLR2, TLR4, and the antimicrobial peptides very well, then the immune system, and particularly the innate immune system, is not going to be functioning very well.

And what I did was to take the various Vitamins D, there are a number of metabolites. I took 1,25 dihydroxy-vitamin D, this is the active metabolite, versus 25 hydroxy-vitamin D. That's the form that the Vitamin D metabolite the sterol is stored in the body with the D-bonding protein in the body fat. And it's stored for very long times. Half life is a month, in some people a year for the storage of 25-D. The body is very good at storing 25-D in between manufacture of it. And then 24,25-D, 25,26-D are just metabolites that occur when 1,25-D is deactivated. And they all lie in the binding pocket. You can see they're essentially coincident, one on top of the other. The tails are a little bit different, but the tails don't have very much to do with the activation of the VDR, certainly with the transcription of the immune mediators that we spoke about.

The only difference between all of these metabolites really is just one alpha hydroxylation and that stabilizes helix 12 and that's known to be essential for the binding to the promoters which allow VDR to do its immune functions.

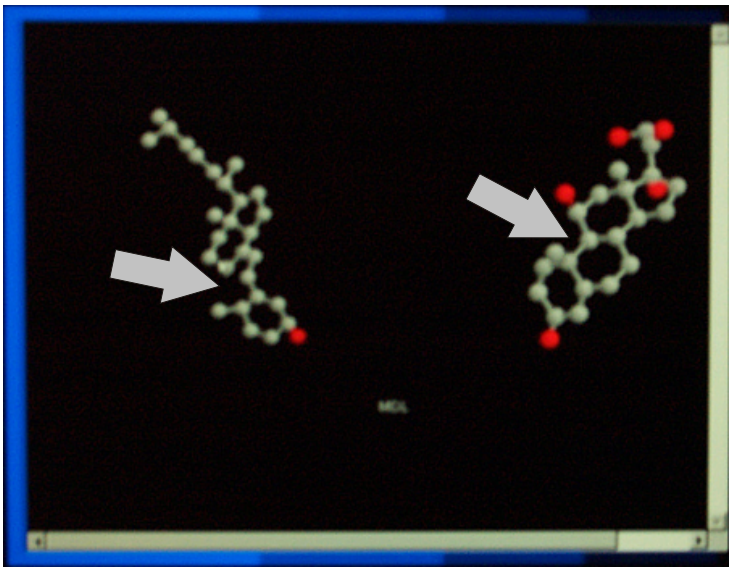
On the right-hand side for those of you that are more technically inclined, I've got a molecule of Olmesartan as it's bound into the VDR ligand binding pocket alongside 1,25-D, and you can have a much closer look at the partial agonism which is produced by that drug in the VDR.

[Modeling: A little bit of basic work...]
27:43 / 1:12/17

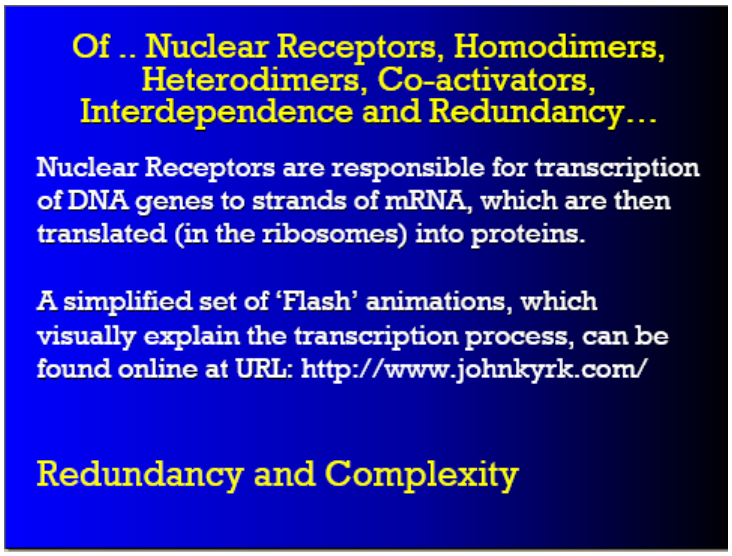
So I'll just do a little bit of basic work here. We've got--on the left we've got Vitamin D which is a secosteroid. And on the right we've got corticosteroid. In this case it's prednisone. (And I've got it upside down. And around the front. Okay, try the side. There we are. Okay.)

So we've got the steroid rings here. You've got the five-member ring, the six-member ring with the methane. Here you've got the five-member ring, six-member ring with methane — it's characteristic of all the sterols. And the only difference is that the

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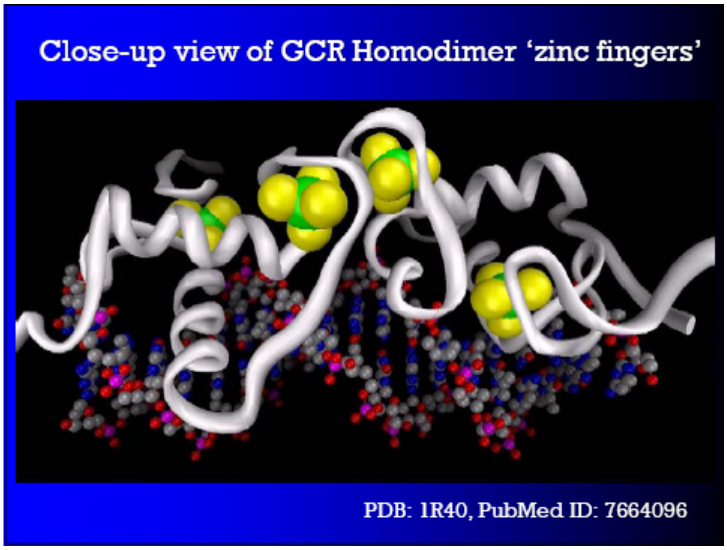
bound between these two carbon which is present here on prednisone and the corticosteriod has in fact been cleaved by an electrocylic reaction, and that makes the Vitamin D a secosteroid rather than a steroid. The interesting thing is that it actually gives it a higher affinity for some of the nuclear receptors because there are more degrees of freedom for this bottom ring because of the inherent flexibility of these two double bonds and the single bond down here. But that's the difference between Vitamin D and prednisone, that one bond.



Of .. Nuclear Receptors, Homodimers, Heterodimers, Co-activators, Interdependence and Redundancy...
29:05 / 1:12/17

I'm going to skip this slide. The main thing to know about the nuclear receptors is that we don't know a heck of a lot about them. We're learning more as every month goes by. But the key two words that I would use to describe how the nuclear receptors work is redundancy and complexity. It's very, very complex.

The receptors combine as homodimers, they combine heterodimers, they can bind with the Retinoid-X receptors and even with the Retinolic acid receptors, and all of these combine to promoter proteins, which then will transcribe different genes from the DNA. Because as you would all know the job of the nuclear receptors is to transcribe the DNA into messenger RNA and then into protein.

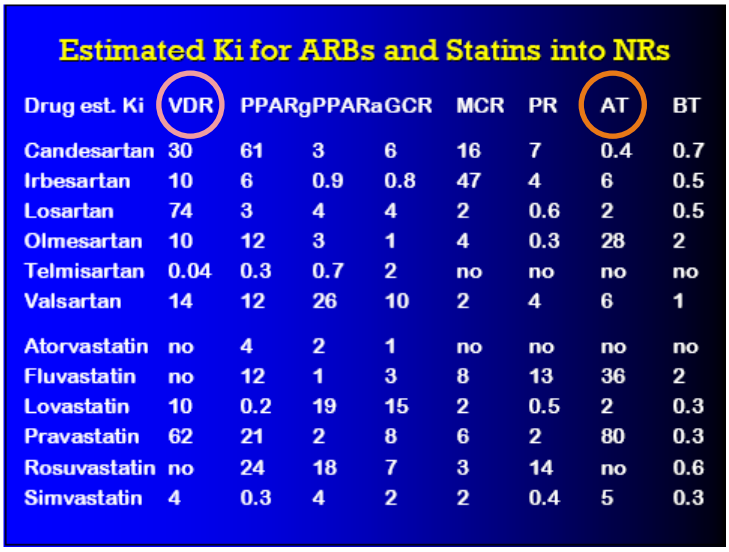


Close-up view of GCR Homodimer 'zinc fingers'
30:00 / 1:12/17

And here we have a closeup of the glucocorticoid receptor Homodimer zinc fingers. That means there are two of these glucocorticoid receptors bound together with the correct promoters, and these zinc fingers are key to the location of the parts of the receptor that actually target the gene and transcribes the gene from the DNA spiral.

You can see here the backbone of the DNA spiral. The base appears across the center. And you can also see the hydrogen bond across the center too that holds the whole structure together. That's a structure that you can download and play on your computer at home, if you're interested.

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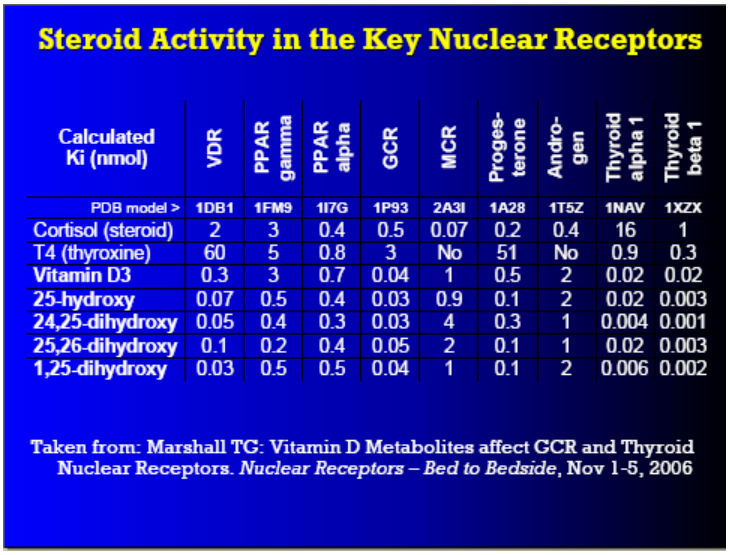
Estimated Ki for ARBs and Statins into NRs
30:51 / 1:12/17

The estimated affinity for the ARBs and statins into the nuclear receptors was what surprised me. And I've got it tabulated here and I'll just take a few data points off this and show you what we're talking about.

Firstly, to answer Dr. Grimes' question in the Lancet, he said that statins, "Are statins analogues of Vitamin D?" he asks.

Well, here's the VDR and if there are going to be analogues of Vitamin D they would have at least have to activate the VDR. And Atorvastatin doesn't have any affinity at all for the VDR, neither does Fluvastatin. Lovastatin has a moderate affinity for the VDR. Pravastatin at normal doses, that is essentially no affinity. Rosuvastatin, no affinity. And Simvastatin a very high affinity. Four nmol Ki indicates that at normal doses Simvastatin would be targeting the VDR.

So the first answer is you can't even talk about the statins as a group because they're very different. Each of the statins is different. And if you take another key receptor, the alpha-Thyroid receptor over here, once again you see Atorvastatin, Fluvastatin has little affect. Lovastatin has a high affect, a very high affinity on the alpha thyroid. Pravastatin and Rosuvastatin is essentially inactive. Simvastatin again has activity in the thyroid subsystem.

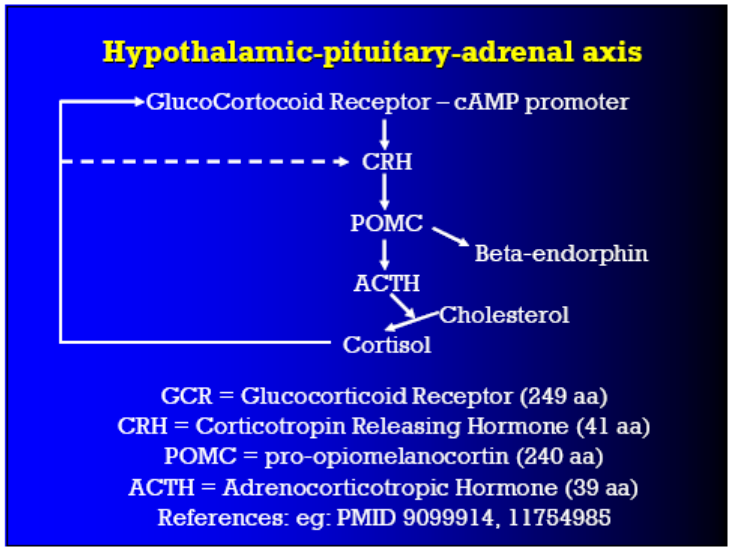


So it's not a simple question are statins analogues of Vitamin D. We've got to break down the degrees of freedom. We've got to talk about which particular drug are we talking about. And then we've got to look at does it have an affinity with Vitamin D. Simvastatin does. So that's a good start.

In the case of the sartans, you've got a similar picture, except the sartans tend to be more homogenous as a group with Telmisartan being the only one that's out there on a limb with an extremely high affinity for the VDR, but it acts as an antagonist for the VDR. It also acts as an antagonist of PPAR-gamma and PPAR-alpha. We wrote a paper last December that goes into that action in more detail.

The Olmesartan that we are particularly interested in, has a wide range of affect across all of the receptors, except the alpha thyroid. It doesn't really target the alpha thyroids very much which is probably a good thing.

I've got similar charts of steroid activity in the key nuclear receptors. I'll just leave that to the PDF and skip that at this point.

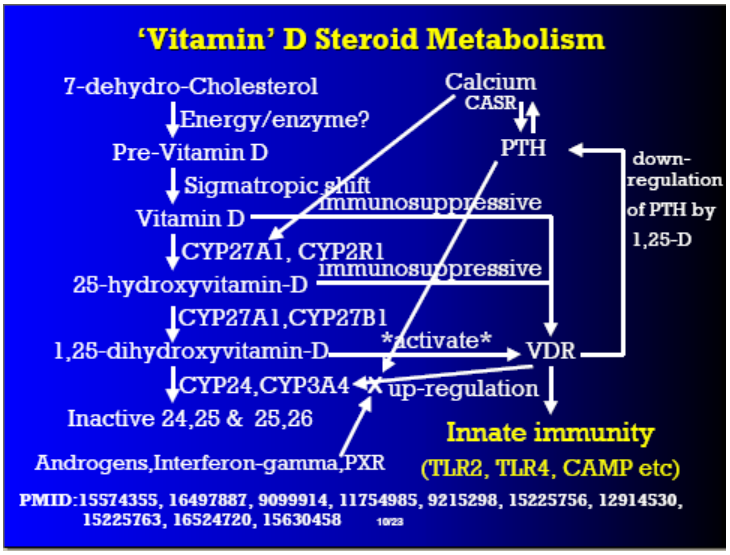


Hypothalamic-pituitary-adrenal axis
33:27 / 1:12/17

I'm going to look briefly at the hormonal control system in the human body. It's pretty well characterized. This is the cortisol access where you've basically got the glucocorticoid receptor with a cAMP promoter. The promoter is known, the GCR as being the transcriber of the gene is gone, and the first metabolite is the corticotropin releasing hormone which is produced from the DNA by the GCR with the cAMP promoter. But then changes to or is converted to Pro-opiomelanocortin, which breaks down into beta-endorphin and ACTH. ACTH then catalyzes the conversion of cholesterol into cortisol and then the cortisol goes around to the top and binds into the glutocorticoid receptor. It also affects the decomposition of corticotropin-releasing hormone, but you have a basic feedback control system there. And the body tends to control all of its hormones, all of its steroids very, very closely.

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If you give a person cortisol and exogenously as an injection, you upset the whole of this balance because you try and bypass the feedback loop. That means you generally have to use much higher doses than are present endogenously within the body itself and you don't get the results you necessarily expected.



'Vitamin' D Steroid Metabolism
34:54 / 1:12/17

Now slightly more complex metabolism is the Vitamin D steroid metabolism and this is pretty well up to date, I think as of October the 23rd [2006], a compendium of everything that's being published and is reliable on the way that the body regulates the hormone called Vitamin D, 1,25-dihydroxy-D, which is what activates the VDR, what allows the transcription of the antimicrobial peptides and the toll-like receptors.

And we start at the top from 7-dehyro-Cholesterol. All of the Vitamins D are produced endogenously in the body from 7-dehydro-Cholesterol. Typically, we think of a vitamin as being something that you have to ingest in order for the body to work properly. But that's not the case with Vitamin D. All of the Vitamins D can be produced from 7-dehydro-Cholesterol and that seems to be the normal way that the body produces it.

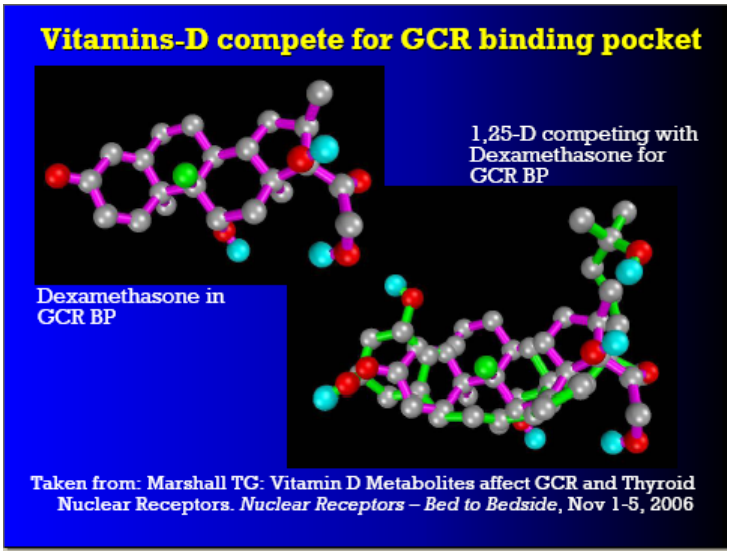
The first step to pre-vitamin D is typically thought to involve UVB energy. However, the electrocyclic reaction — conrotatory electrocyclic reaction — is found elsewhere in nature, catalyzed by enzymes. So there's probably some question marks there that we still have to go looking for. Do we need UVB or do we need enzymes? Big question mark over the whole concept of whether Vitamin D is a vitamin or whether it is just a hormone precursor.

And then pre-vitamin D the electrons rearrange slightly in a sigmatropic shift to create vitamin D which then is hydroxilated to 25-hydroxyvitamin, the single hydroxy by CYP27A1— That's one of the p-450 enzymes or CYP2R1. And then that, monohydroxy, is converted to the dihydroxy by CYP27A1 again — a heme-related enzyme — or by CYP27B1. And B1 is primarily expressed in the macrophages, in the monocytes, in the phagocytes and it converts 25-D to 1,25-D, but it doesn't go all the way back to the sterol system and that's the one which is most active in the immune system.

Then finally, 25-D is degraded into an inactive form by CYP24 and CYP3A4 which are up regulated by the VDR. So if there's too much 1,25-D, the VDR upregulates the inactivation pathway and that upregulation is cancelled or offset by androgen activity: testoterone, estrogen, interferon gamma; and that's why interferon gamma and 1,25-D are so closely coupled together because interferon gamma stops 1,25-D from being degraded. It stops the enzymatic feedback there and also by PXR. And when the VDR is activated you have innate immunity and you have down-regulation of the parathyroid hormone by the 1,25 dihydroxy-D — 1,25 dihydroxy-vitamin D — and then the parathyroid hormone in conjunction with the calcium sensing receptor which is in the kidneys sorts out the body's calcium homeostatis and then the body's calcium homeostatis feedbacks into the expression of CYP27A1. And there's your feedback path for the Vitamin D steroid metabolism.

Not simple by any means, and actually not unreasonable if you consider that the immune system would have had to adapt through evolution in many stages and that's really what we're seeing as we dig further and further into the way that the Vitamin D metabolism works in man.

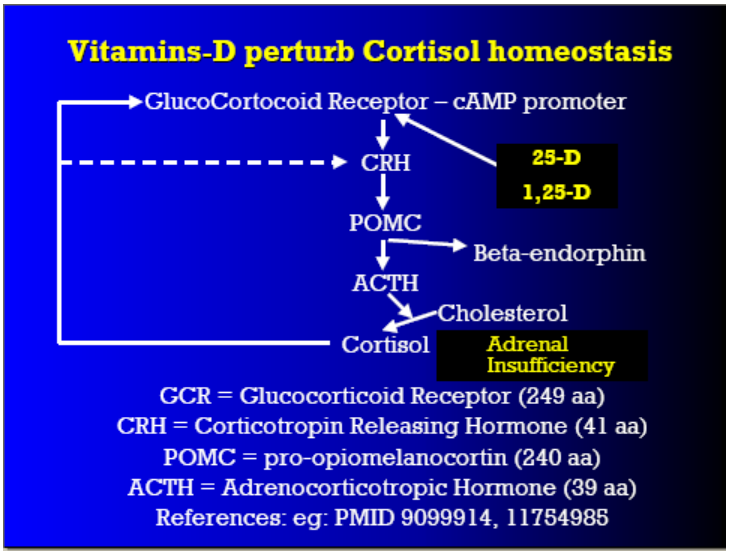
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Vitamins-D compete for GCR binding pocket
38:57 / 1:12/17

Well, up on the top left here I've got a configuration of dexamethasone as it stops into maximum affinity and to the glucocorticoid receptor binding pocket — ligand binding pocket. And the backbone is in purple. Here is the fluorine atom [green] and the oxygen is obviously in red. And on the lower right I've got 1,25D superimposed on that, with a green backbone, showing how it binds into the GCR and it binds into exactly the same ligand binding pocket. There is no doubt that 1,25D displaces cortisol and dexamethasone from the GCR in a concentration-dependent manner and vice versa of course.

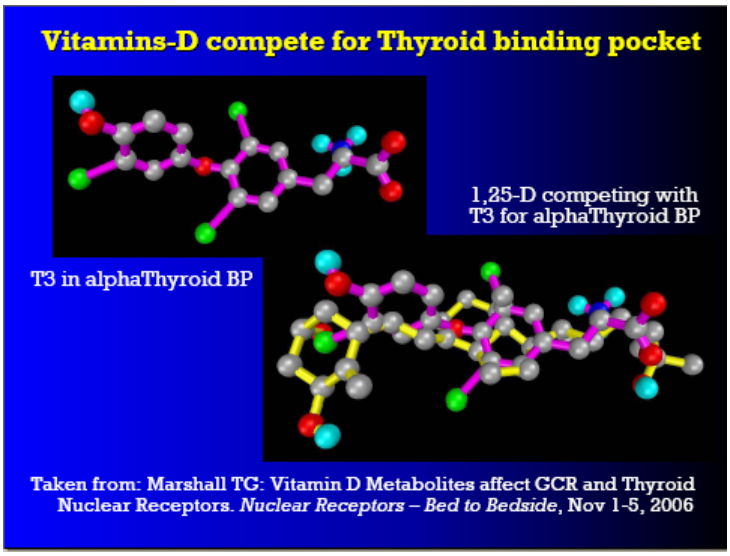
So when you factor this knowledge in, what happens when the immune system run haywire and 1,25D levels build as we see in clinical practice?



Vitamins-D perturb Cortisol homeostasis
39:54 / 1:12/17

25-D and 1,25-D go into the glucocorticoid receptor and intercede — make this whole hormonal feedback system break down — and you end up with patients who have adrenal insufficiency which is very, very common in these Th1 diseases.

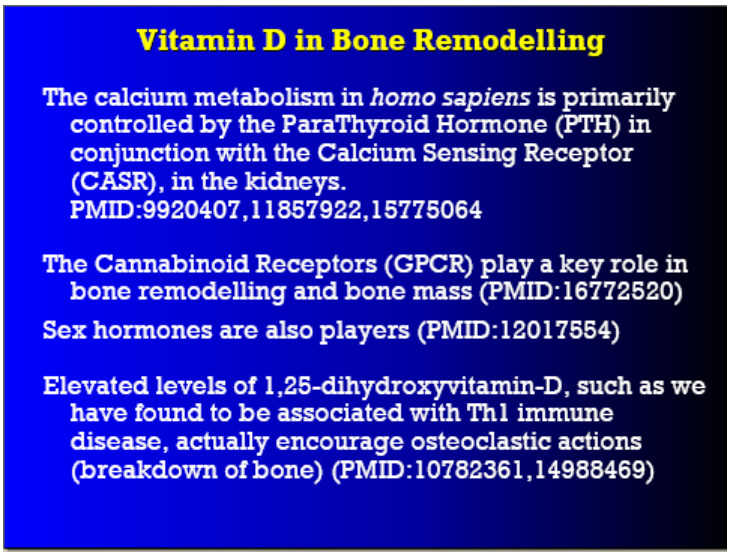
However, I point out that increasing the dose of cortisol to try and cope with the adrenal insufficiency is the incorrect way to go about treating the problem. It has to be attacked at the actual cause by lowering the 25-D, the ingested Vitamin D, and the 1,25-D, the inflammatory load.



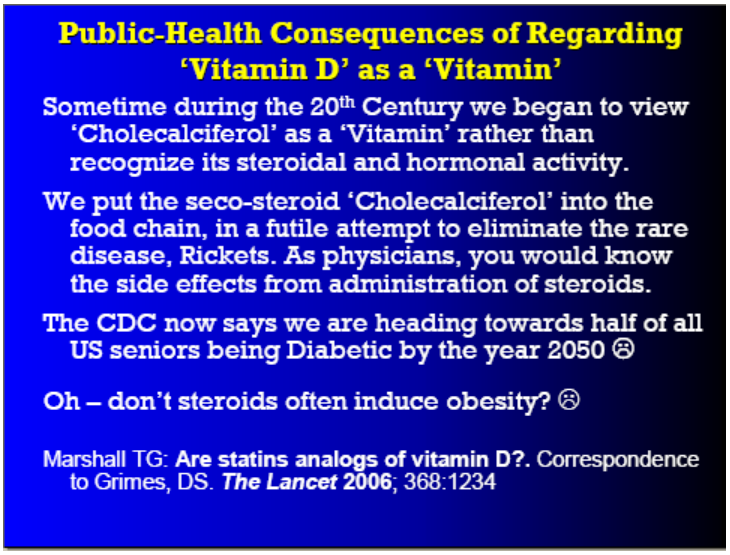
Vitamins-D compete for Thyroid binding pocket
40:39 / 1:12/17

However, the Vitamin D also competes for the alpha-1 Thyroid binding pocket, and hypothyroidism is very common in these Th1 diseases. Here's T3. You can see the three atoms here in green. T3 as it binds into the alpha-1 Thyroid receptor. And here we have 1,25D competing with T3 for the ligand binding pocket of the alpha-1 thyroid receptor. And as you can see they will displace each other from the receptor in concentration and affinity-dependent manners.

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Vitamin D in bone remodeling I'll skip in this group because I'm running behind time.



Public-Health Consequences of Regarding 'Vitamin D' as a 'Vitamin'
41:26 / 1:12/17

But I do want to briefly consider the public health consequences regarding Vitamin D as a vitamin.

If we look back to the 1923 nobel oration the 1923 nobel prizes given for the elucidation of the function of the sterols in particularly what we know as Vitamin D in the body and if you look at the nobel oration, you'll find that there really no concept of vitamin in it. Originally, the concept was this is a sterol. This is a sterol, a secosteroid, not a vitamin.

Sometime during the 20th Century we began to view Cholecalciferol as a vitamin rather than focus on its steroidal and hormonal activity. And when we started putting this in the food chain, in a futile attempt to eliminate the rare disease rickets — rickets now turned out to be in the latest, very comprehensive studies to be eliminated only by calcium and phosphorous in the diet. But we put it into the food chain back starting about 1950s to try and eliminate rickets and we haven't eliminated rickets, although the incidence has dropped.

As physicians, many of you who are physicians would certainly know the side effects from the administration of steroids. The Centers for Disease Control in the USA says that the US is heading towards half of all US seniors being diabetic by the year 2050. That is exactly the track that the US population is on right now. And we all know about obesity.



REUTERS
43:01 / 1:12/17

Here's an article from earlier this week. *"U.S. Children grow bigger bellies,"* from Reuters. Startling data.

Actually over here [left column under picture] it's interesting. Canadians getting fatter but more slowly. But what they found was that this particular study that Rochester School of Medicine found was that ten and a half percent of boys and girls had too much abdominal fat in1999, as measured by waist circumference. This included seventeen and a half percent of boys and girls in 2004. In just five years, the obesity rose sixty-five percent.

Now I don't think McDonalds sales rose sixty-five percent in those same five years from 1999 to 2004. There were other factors at play. Obviously I have my theories as to what those other factors are.

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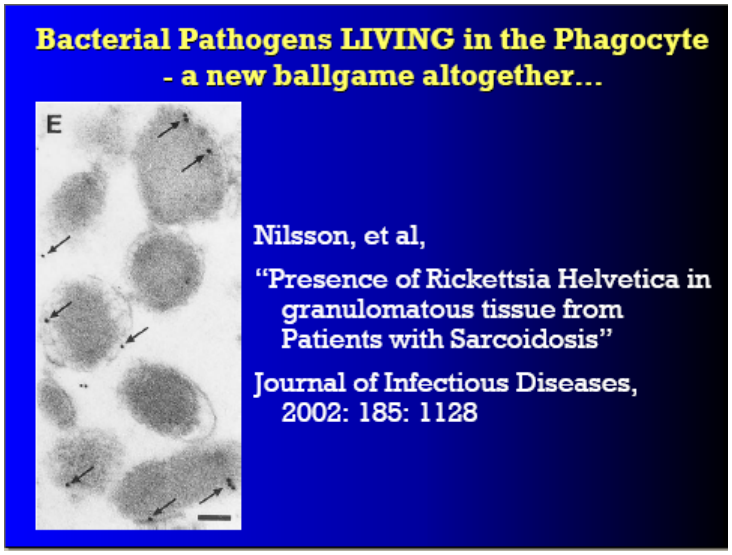
[OTHER FACTORS]
43:52 / 1:12/17

Here they are. These are the US experience and I've put this slide together because in Australia you've all been pretty well shielded at this point from supplementation of the diet with exogenous Vitamin D, artificial Vitamin D.

In America, milk and yogurts are fully supplemented with Vitamin D. If you have two glasses of milk a day you get the entire supposed RDA of Vitamin D, and we find that that is more than enough to take the levels of 25-D in the body into immunosuppressive range.

Cheese, particular cheese slices that are intended for kids' meals, for kids' lunches, are fortified with Vitamin D in the US. Kraft Macaroni and Cheese and all of the cereals, all of the breakfast cereals for kids, they're all fortified with Vitamin D. The kids can't get away from this stuff. And now of course orange juice is fortified with Vitamin D to make it really good for you.

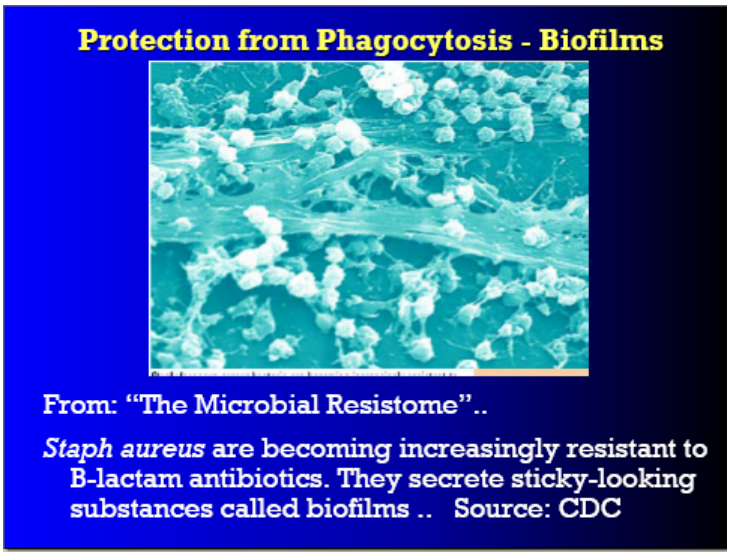
That's the experience in the United States. It's a very sad experience, but that's what's happening at the moment. And I put at the bottom right there a bottle just to remind us, but the calcium for Vitamin D is so often prescribed to patients who present with these inflammatory disease presentations, diagnoses, and who also have osteopenia. And that's counterproductive because the people that need the Vitamin D least are the ones that get supplemented and that's purely because clinical medicine is not measuring the correct metabolite. They need to measure 1,25 dihydroxy, the active metabolite, as well as the inactive metabolite.



Bacterial Pathogens LIVING in the Phagocyte
45:41 / 1:12/17

So let's go on to talk about the pathogens which is the other half of the disease equation.

Now we've known for sometime that there are bacterial pathogens that are capable of living in the phagocytes. This is from a study "Presence of Rickettsia Helvetica in granulomatous tissue from Patients with Sarcoidosis," 2001 — late 2001 this came out. And it showed bacteria staining inside the phagocytes. The authors inferred that these bacteria were rickettsia and they may very well have been, but the point was bacteria can survive inside the phagocytes. **That is a mind boggling concept**. The phagocyte is supposed to phagocytose they are supposed to breakdown the bacterial DNA and render the bacteria harmless.



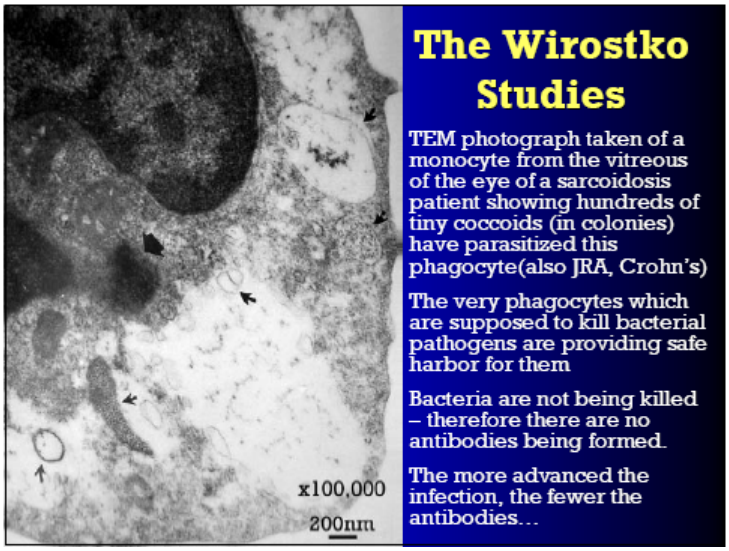
Protection from Phagocytosis — Biofilms
46:42 / 1:12/17

This is a photo from the paper, the review paper, The Microbial Resistome. This talks a lot about horiozontal transfer of DNA. Search for it PubMed or in Google, The Microbial Resistome. It's brillant.

The reason I put it on there is to show biofilms. This is from the Centers for Disease Control and it shows how Staph aureus produce biofilms which are a sticky looking substance. They secrete sticky looking substances called biofilms.

And that's what we see associated with these tiny bacteria that are found in the inflammatory diseases.

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The Wirostko Studies
47:13 / 1:12/17

The Wirostko Studies were performed in the late 1980s at Columbia University in New York by the Wirostkos, the family of Wirostkos and another collaborator. And what they did was quite unique. They took patients who had Crohn's disease, juvenile rheumatoid arthritis, and sarcoidosis and they used transmission electron microscopy to examine the phagocytes and they stained the bacteria.

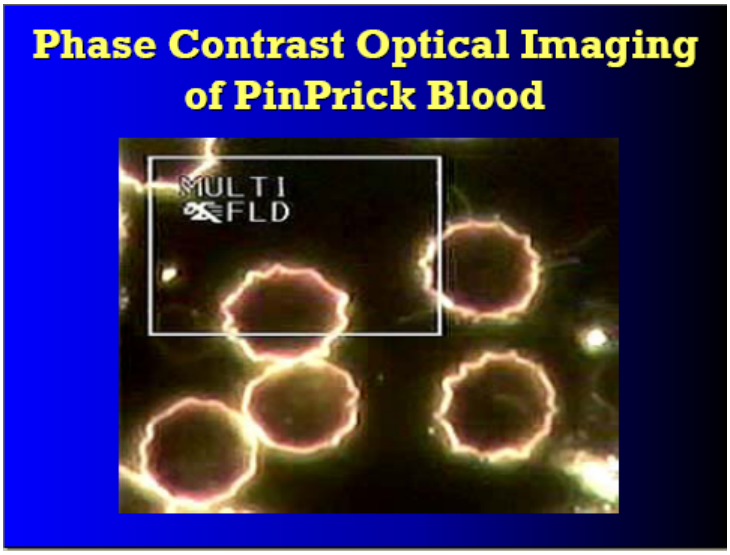
They were particularly interested in seeing whether it was possible for phagocytes to become infected. And they found that all of the phagocytes — monocytes, macrophages, lymphocytes, and neutrophils — were all infected by these tiny little L-form bacteria that were first described in 1934 by ME Kleinberger Nobel of the Lister Institute.

And we have here a single isolated coccoid about .01.015 microns diameter. What am I saying, microns? Nanometers in diameter. Ten to fifteen nanometers diameter.

And then we have a kidney-shaped colony which in itself I haven't counted these but they must well be over a hundred individual coccoids in there, string of about a dozen coccoids in here, and another gaggle of coccoids there, and another artifact of stained bacterium there. These are tiny little bacterium.

And what was particularly interesting about this photo was this crenation in the nucleus which doesn't show too well here, but the nucleus, part of the nucleus is actually breaking off as shown by this dark arrow. It's shown by the big arrow. And that nuclear activity was close to the largest colony of bacteria in this case.

Every one of these photographs that they took showed infection and every one of the photographs that they took show slightly different physical manifestations of an infection in some case vastly different. But the two things that were common throughout them all was a lot of transparent and semi-transparent material like this, the biofilm, and also the tiny, tiny coccoids.



VIDEO, optical microscopy
49:44 / 1:12/17

Now is a phase contrast optical microscopy. This is done with a Bradford Microscope. It has also been done with quite inexpensive microscope, microscopic equipment. I'll just stop it there, pause it.

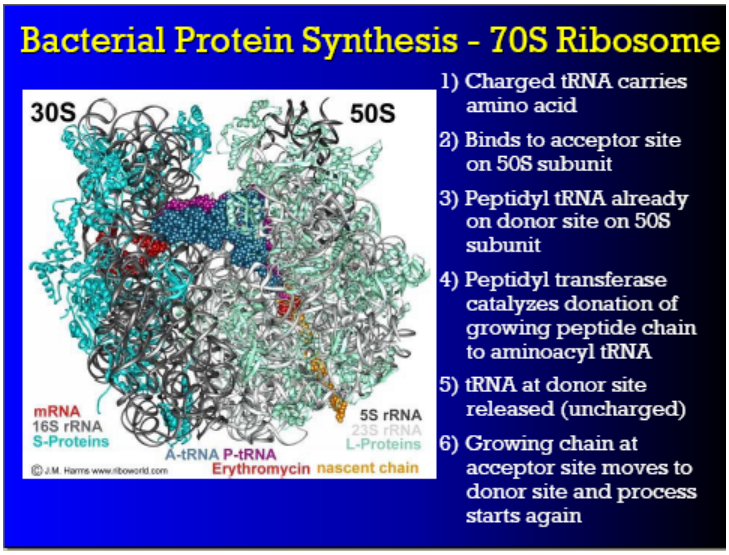
And what we've got is these tiny little threads, thread-like appendages, which are made of a translucent tubular material which stains with fluorescent antibodies for bacterial RNA. (How did I get back there?) And these are exiting a crenating red cell in this case.

This is a technique which was devised by Andrew Wright in Northern UK where he takes pinpricked blood, puts it between a slide, seals air out with vaseline and then allows the blood to degrade a period of six to thirty-six hours. And as you can see this is a monocyte that has disintegrated. You can see these tiny long forms that have no analogue in pathology. There is no explanation in pathology for what these are, except of course that they are the bacterial L-forms.

(Video continues of biofilm moving very different from Brownian elements moving in background, and static cells.) This particular picture there is a bacterial L-form here which is going to do a linear movement toward the bottom, just to show those of you that think it's all Brownian in motion. It is not. It's going to gradually

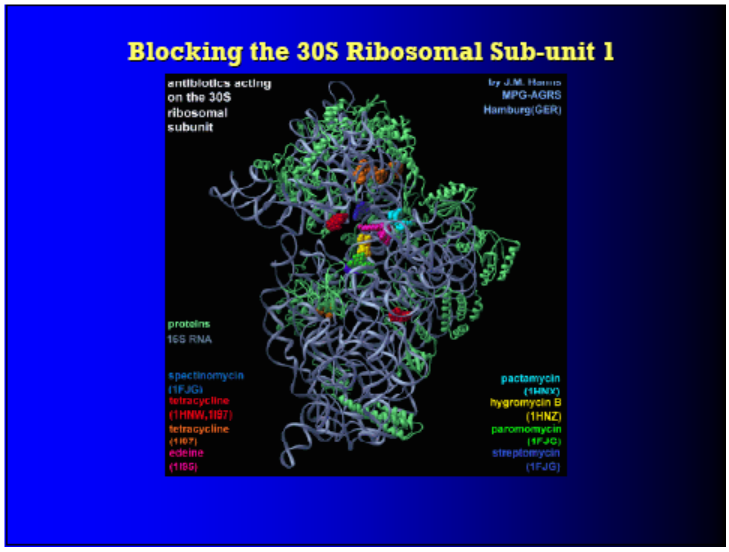
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work to the bottom, as I speak, but this equipment, the equipment that was used to produce this photograph, was bought off E-Bay, very, very simple analogue microscrope x400 and a standard camera with computer software to amplify up the size of the image. So this little fellow will go off the screen and I'll just allow it to refocus to show you that it keeps going down and eventually disappears totally from the field of view. There is Brownian motion visible and something like Brownian motion in the other particles there, but this thing seems to know where it's going.



Bacterial Protein Synthesis — 70S Ribosome
52:17 / 1:12/17

Okay. So what do our antibiotics do? Our antibiotics go after the bacterial ribosomes. The 70S bacterial ribosome is in two halves, the 30S, 50S subunits. The 30S, the MRNA is translated by the helical structures at the top. You've got the tRNA here which donates the amino acids to the growing protein chain. And then the protein leaves the bottom of the 50S section. And the antibiotics we use target different areas of the ribosomes.



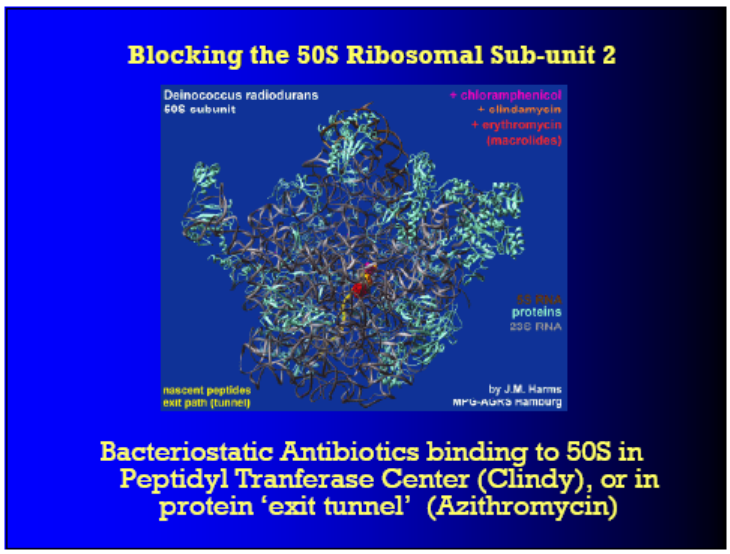
Blocking the 30S Ribosomal Sub-unit 1
52:49 / 1:12/17

If we look up at just the 30S subunit, what we've got is up top these helical structures which involve with advancing the mRNA as it is decoded.

And right sitting there are the molecules of tetracycline in the various locations that they bind with quite strong affinity.

This work is from the Max Plank Group in Hamburg, Germany. Max Plank Ribosome Group.

And so minocycline, demeclocycline, the first of the antibiotics we use inhibits — it doesn't stop — it inhibits, it slows down the translation of the mRNA at that point, the top of the 30S ribosome.



Blocking the 30S Ribosomal Sub-unit 1
52:49 / 1:12/17

Then the 50S ribosome, you've got the protein exit channel here at the bottom and in red here, that's where the macrolides bind the erythromycin and the azithromycin that we use. And just above that is where the clindamycin binds. If you'll remember that's the third antibiotic we use in the longer term cocktail to totally get rid of the bacterial load that the patients are carrying.

And the important thing to note from the animation is that each of these antibiotics is symbiotic. None of them interfere with each other. The addition is linear and it's symbiotic.

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Plasmids Make for a Toxic 'Pea-Soup'

367 Microbial Genomes have now been sequenced, and another 619 have been partially completed.

It has become obvious that most Bacterial species are not homogenous, consisting not only of a single chromosome, but also possessing a variable number of self-replicating plasmids carrying DNA, and Genes.

Borrelia burgdorferi has a large number of plasmids, indeed, nearly half its genome exists on its 21 self-replicating plasmid sub-units

Yet even common species, like *Staphylococcus epidermidis* ATCC 12228, give rise to plasmids (*S. Epidermidis* has 10% of its DNA spread over six self-replicating plasmids)

These plasmids are not targeted by antibiotics, and, unless destroyed by the immune system, will persist in chronic intra-cellular infections – think of it as a 'DNA Pea-Soup'

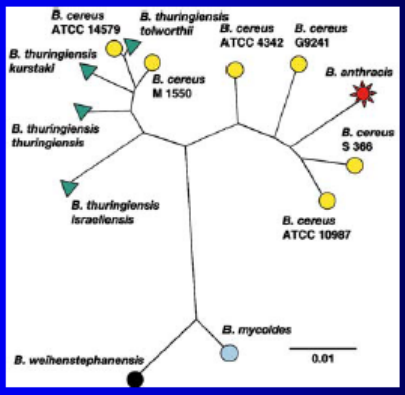
Plasmids Make for a Toxic 'Pea-Soup'
52:49 / 1:12/17

Now what about the actual pathogens? Still everybody wants to know well what species is it; what species causes these infections?

The answer is: You're dealing with a chronic infection building over a lifetime. You're not going to be dealing with a species. You're going to be dealing with massive amounts of horizontal transfer of DNA, particularly of plasmids.

And now that we know what the genomes look like we've got some bacteria, for example, borrelia burgdorferi, that has gotten nearly half its genome on 21 self-replicating plasmid subunits. It is most definitely going to be sharing plasmids. And even common species like staph epidermidis have ten percent of the DNA split over six self-replicating plasmids. Those plasmids can easily be shared. They are not targeted by antibiotics and they are only targeted by the innate immune system and then only weekly. They are going to accumulate over time unless cleared by the immune system and you're going to be dealing not with UNI-microbial disease but with a POLY-microbial disease. A combination of the infectious history of the individual including both viral and bacterial pathogenic challenges.

Plasmids transmit DNA 'horizontally'



Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax
Hoffmaster, Alex R. et al. (2004) Proc. Natl. Acad. Sci. USA 101, 8449-8454

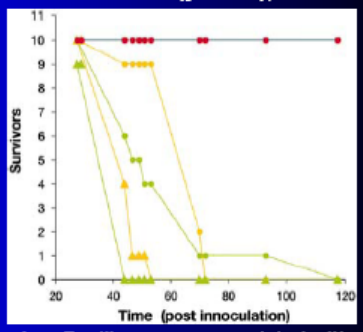
Plasmids transmit DNA 'horizontally'
55:29 / 1:12/17

And here is a paper from the National Academy of Sciences, 2004, where two plasmids from bacillus anthracis were transferred to the genome of bacillus cereus and a patient died very quickly as a result of that. They actually were wondering why the patient had died from bacillus cereus until they noticed that, that particular genome had also acquired at some point the two plasmids from bacillus anthracis. And those two plasmids, one conveys the ability to evade phagocytosis, the other conveys the ability to kill people.

And particularly interesting over here is bacillus thuringiensis. That's used as an insecticide. That's sprayed onto our crops as an insecticide. That's very close in phylogeny.

Plasmids transmit DNA 'horizontally'

Fig. 4. Survival of mice i.p. challenged with *B. cereus* G9241 hybrid (green), *B. anthracis Sterne* (yellow), and *B.cereus* ATCC10987 (red)

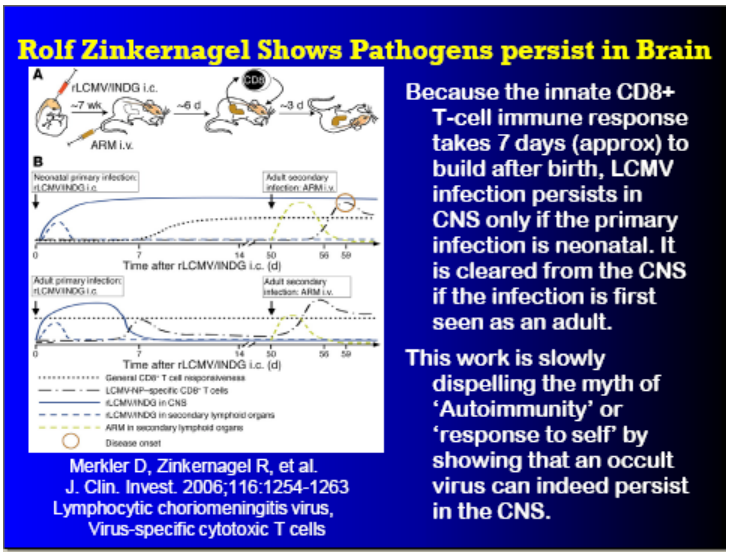


Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax
Hoffmaster, Alex R. et al. (2004) Proc. Natl. Acad. Sci. USA 101, 8449-8454

Plasmids transmit DNA 'horizontally' [continued]
56:31 / 1:12/17

Now this is survival of mice challenge with that particular hybrid of cereus anthracis plasmids. And normally it's harmless. That's anthracis itself [refer to chart] whereas the hybrid kills more quickly than anthracis itself. The horizontal transfer of DNA is absolutely key to understanding the pathogens in these diseases.

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Rolf Zinkernagel Shows Pathogens persist in Brain
57:07 / 1:12/17

My final slide is work from Rolf Zinkernagel who together with Peter Daughtery at John Curtain did a lot of study on antibody presentation and got the 1996 Nobel prize for it.

Now Rolf has dedicated his life subsequent to the Nobel to showing that autoimmune disease is not due to antibodies themselves. And I had a very good long discussions with him at Karolinska [DMM 2006].

And this particular experiment which is a recent publication by his, 2006, they injected lymphocyctic choriomeningitis virus into newborn mice, into the brain of newborn mice. And what they found was that the virus appeared to be cleared and it couldn't be detected by normal techniques, but it was clearly persistent in the brain. Because when those mice were challenged later in life with either a slightly different virus or with the same virus, then there was a very energetic immune reaction and the mice died. Whereas, if the mice had been initially challenged later in life after the adaptive immune system had started to work properly then they were able to cope with both the initial and subsequent challenges from the virus.

Because, you see, mice are just like human beings. When they're born they only have innate immunity. They don't have adaptive immunity running. In the case of mice you can see the T cells starting to become active here at about day seven in their lifespan. In humans, it's a little bit longer than that. It's in weeks. But it takes a while. And during that time the infant is only protected by the innate immune system. And what happens in that time is quite critical.

And what Rolf's group has shown is that you can have an occult virus which is persisting in the brain through life which is later activated into an extremely energetic and pathogenic state.

That is a model for what we're also seeing with the bacterial pathogens in these chronic diseases.

So there we are. Thank you very much for listening to the talk. And we'll see if there are any questions.

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Q&A

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