

Immune response inversion after hyperimmunisation

Possible mechanism in the pathogenesis of HLA-linked diseases

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SUMMARY The crosstolerance hypothesis suggests that animals sharing antigens with some microorganisms will produce low antibody levels in the early part and high levels in the latter part of an infection. Antibody responses have been measured in high responder B10.M and B10.D2 mice and low responder C3H and A.Thy-1.1, as well as F1 hybrids (B10.M × A.Thy-1.1) and (B10.M × C3H/He), after repeated immunisation with the antigen ferritin, involving altogether 483 mice. An inversion in the immune response was found to occur and similar delayed high antibody responses have been described in rheumatic fever. It is suggested a mechanism of immune inversion may operate in the pathogenesis of HLA and blood group-linked diseases.

The ability to produce specific immune responses (IR) to many different antigens, has been shown to be under genetic control (Benacerraf and McDevitt, 1972) while an increased susceptibility to disease has been described in subjects carrying defined genetic markers. For instance, subjects carrying HLA B27 have a greater susceptibility of developing ankylosing spondylitis (Brewerton *et al.*, 1973; Schlosstein *et al.*, 1973), while carriers of blood groups A and B or secretors of Lewis A substance have a greater chance of developing rheumatic fever (Glynn, 1975).

The mechanism mediating high antibody responses in animals or increased susceptibility to disease in subjects carrying defined genetic markers, is at present unknown. However, two theories have been proposed to explain this genetic association. (1) 'Two gene theory', or linkage disequilibrium hypothesis. (2) 'One gene theory', or crosstolerance hypothesis or molecular mimicry theory.

The 'two gene theory', or linkage disequilibrium hypothesis, states that associated or linked with the marker gene (first gene) is another gene (IR-gene) (second gene) whose gene product mediates a high antibody response in high responder animals and in humans. The linked or associated second gene, located in the 'disease susceptibility' locus, codes for

a gene product which is responsible for an increased incidence of the disease (McDevitt and Bodmer, 1974).

However there are several problems with this theory. No IR-gene product or disease susceptibility locus has so far been identified. No mechanism has been proposed to explain how the putative gene product contributes to an increased incidence of the disease. The progressively increasing number of specific IR-genes linked to the 'major histocompatibility complex' or the number of different diseases linked to HLA, raises the problem of an ever expanding pool of genes which have to be accommodated within the restricted genetic space in and around the H-2 or HLA complex.

The 'one gene theory' or crosstolerance hypothesis or molecular mimicry mechanism (Cinader, 1963; Damian, 1964; Snell, 1968) is an alternative and simpler way of explaining IR-gene phenomena and HLA-linked diseases (Ebringer, 1978). The central theme of the 'crosstolerance hypothesis' is that low responders produce a low antibody response because its transplantation or self antigens cross react more with the test antigen than do the self antigens of the high responder animals. In the 'crosstolerance hypothesis', the marker gene alone is responsible for the low response or the increased susceptibility to disease, hence the name of 'one gene theory'. Evidence in support of such a mechanism has been obtained for the TGAL (Ebringer and Davies, 1973)

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and ferritin (Deacon and Ebringer, 1977) IR-gene systems.

In genetically associated diseases the cross-tolerance hypothesis implies that there is molecular similarity between HLA, blood group, or salivary Lewis substance and some bacterial antigens against which the patient mounts a poor immune response because it partially recognises the micro-organism as a self structure. Thus the micro-organism is able to proliferate and furthermore any antibodies produced will not only have antimicrobial activity but also some antiself or autoimmune activity. Continued presence of the micro-organism would lead to further production of cross-reactive antimicrobial antibodies, which would bind to self antigens, activate the complement cascade, and produce local inflammation at a site distal and quite removed from the site of infection.

The crosstolerance hypothesis thus proposes a poor immune response in the early stages of the infection but with continued presence of the micro-organisms, delayed and high antibody responses in the latter part of the infection which could be responsible for such chronic inflammatory disorders as rheumatic fever or ankylosing spondylitis.

If IR-gene systems operate by similar mechanisms as HLA-linked diseases, then a low responder animal should, on prolonged exposure to antigen, also show delayed high antibody responses as suggested for HLA and blood group-linked diseases.

The genetic control of the immune response to ferritin has been investigated in mice (Young *et al.*, 1976, 1977). It was found after primary immunisation with ferritin in phosphate-buffered saline (PBS) that A.Thy-1.1 and C3H mice are low responders and B10.M and B10.D2 mice are high responder animals (Table 1).

Table 1 Antibody response of mice immunised with horse spleen ferritin

Percentage antigen bound of 2.5 ng ¹²⁵ I-ferritin*				
Strain	No. animals	Mean % bound	± SE	Significance
B10 M	10	51.07	1.21	P < 0.001
B10.D2	9	31.77	3.70	P < 0.001
A.Thy-1.1	9	1.22	0.65	P < 0.001

µg Ferritin bound/ml serum†				
Strain	No. animals	Mean µgAG bound/ml	± SE	
B10.M	27	687.1	140.7	P < 0.001
A.Thy-1.1	26	48.5	7.4	P < 0.001

*Mice were immunised intraperitoneally with 200 µg ferritin in PBS and bled out 14 days later (Young *et al.*, 1976).

†Mice were immunised intraperitoneally with 400 µg ferritin in PBS and bled out 20 days later (Young *et al.*, 1977).

We have now further investigated the immune response to ferritin in both high responder B10.D2 and low responder A.Thy-1.1 mice, as well as some other strains using altogether 483 mice. Repeated administration of ferritin has been used to try to reproduce the situation of a self-replicating antigen such as that of a proliferating micro-organism, during a prolonged infection in a vertebrate animal.

Materials and methods

ANTIGEN

Horse spleen ferritin (2 × crystallised) was obtained from Miles Ltd. (England).

ANIMALS

All the mice used in these experiments were aged between 10 and 12 weeks and were bred and maintained in our laboratories.

IMMUNISATIONS

The mouse strains B10.D2 and A.Thy-1.1 were sequentially immunised with ferritin over a 70-day period. On day zero, 90 mice from each strain were immunised with 200 µg ferritin in 0.1 ml incomplete Freund's adjuvant (Difco, Michigan, USA) in a single intraperitoneal site. 14 days later all the test mice were boosted with 200 µg ferritin in 0.1 ml PBS intraperitoneally, and thereafter once every 7 days until day 70. Before each immunisation, 10 mice from each strain were bled out by cardiac puncture, serum separated, and stored individually at -20°C. The detailed immunisation and bleeding protocol is shown in Table 2.

The mouse strains B10.M, A.Thy-1.1, C3H/He, and hybrids (B10.M × A.Thy-1.1)F1 and (B10.M × C3H/He)F1 were hyperimmunised with horse spleen ferritin over a 74-day period. On day zero, between 30 and 70 mice from each strain and F1 hybrid were immunised with 200 µg ferritin in 0.1 ml complete Freund's adjuvant (Difco, Michigan, USA) in a single intraperitoneal site. 14 days later all test mice were boosted with 200 µg ferritin in 0.1 ml PBS intraperitoneally, and thereafter once every 10 days until day 74, when all mice including 10 control mice per strain were bled out by cardiac puncture, serum separated, and stored individually at -20°C.

SEPARATION OF IGM AND IGG FERRITIN ANTIBODIES

To 2 ml of pooled B10.D2 or A.Thy-1.1 antiserum, obtained after four immunisations with ferritin, was added 2 ml saturated ammonium sulphate at pH 8 (with 880 ammonia, BDH Ltd.), kept at 4°C for one hour and samples were then centrifuged at

Table 2 Immune responses to ferritin in sequentially immunised A.Thy-1.1 and B10.D2 mice after primary immunisation in incomplete Freund's adjuvant (IFA)

Day	Immunisation protocol	Number of test mice		Mean (\pm SE) percentage bound of 100 μ g 125 I-ferritin by 20 μ l undiluted serum		Mean (\pm SE) percentage bound of 250 μ g 125 I-ferritin by 10 μ l undiluted serum	
		B10.D2	A.Thy-1.1	B10.D2	A.Thy-1.1	B10.D2	A.Thy-1.1
0	200 μ g IFA						
14	200 μ g PBS	14	10	11.68 \pm 0.77	15.65 \pm 1.60	ND	ND
21	200 μ g PBS	9	10	10.12 \pm 1.54	40.61 \pm 5.59	"	"
28	200 μ g PBS	9	10	10.74 \pm 1.80	54.75 \pm 4.32	"	"
35	200 μ g PBS	9	10	11.21 \pm 2.30	78.52 \pm 4.98	"	"
42	200 μ g PBS	9	10	14.14 \pm 1.98	67.37 \pm 5.33	"	"
49	200 μ g PBS	9	10	14.05 \pm 1.51	83.98 \pm 1.63	"	"
56	200 μ g PBS	9	10	11.86 \pm 2.52	84.67 \pm 1.89	"	"
63	200 μ g PBS	8	9	21.50 \pm 3.99	81.77 \pm 3.56	"	"
70		8	9	ND	ND	6.97 \pm 2.84	46.40 \pm 2.05

ND = not done.

10 000 rpm for 15 minutes. Supernatants were discarded, precipitates dissolved in 2 ml PBS, were dialysed against three 5 litre changes of 0.002 M sodium phosphate buffer, pH 6.0 (with 1N HCl), at 4°C for 72 hours, the IgM macroglobulin fraction precipitating out leaving the IgG fraction in solution. The precipitate was washed three times with 0.002 M sodium phosphate buffer, pH 6.0, and dissolved in 3 ml PBS. The IgG solution obtained after dialysis was diluted into 3 ml PBS and antibody determinations carried out on both fractions.

ANTIBODY DETERMINATIONS

Ferritin antibody estimations in mouse sera and immunoglobulin fractions were carried out using an antigen excess technique described in detail previously (Young and Ebringer, 1976; Young *et al.*, 1977). Briefly, to 10 or 20 μ l of mouse serum was added 100 or 250 μ g ferritin, labelled with 125 I using the chloramine-T method (Hunter, 1969) and antigen-antibody complexes precipitated with excess rabbit antimouse immunoglobulin serum. Isotopic estimations of the precipitates were carried out and results expressed as the percentage of ferritin bound by each antiserum.

DOMINANCE INDEX ESTIMATION

The Fisher dominance index (D) is an estimate of the phenotypic penetrance of a character in a population (Falconer, 1960) and is given by

$$D = \frac{2(F1 - L)}{(H - L)} - 1$$

where H, F1, and L are the quantitative antibody responses of the high, F1 hybrid, and low responder animals respectively. It is apparent that for a dominant trait, the dominance index takes a value of +1, for a codominant trait the value of zero, and for the situation where low response is dominant over high response, the value of -1.

Results

ANTIBODY RESPONSES AFTER HYPERIMMUNISATION

The immune response to ferritin after primary immunisation in incomplete Freund's adjuvant and 8 sequential immunisations in PBS, in B10.D2 and A.Thy-1.1 mice is shown in the Fig. and Table 2. The Fig. shows that after primary immunisation in incomplete Freund's adjuvant there is very little difference between the quantitative immune response of B10.D2 and A.Thy-1.1 mice. As the number of immunisations is progressively increased, it becomes apparent that A.Thy-1.1 mice produce a much higher quantitative antibody response to ferritin than do B10.D2 mice ($P < 0.001$).

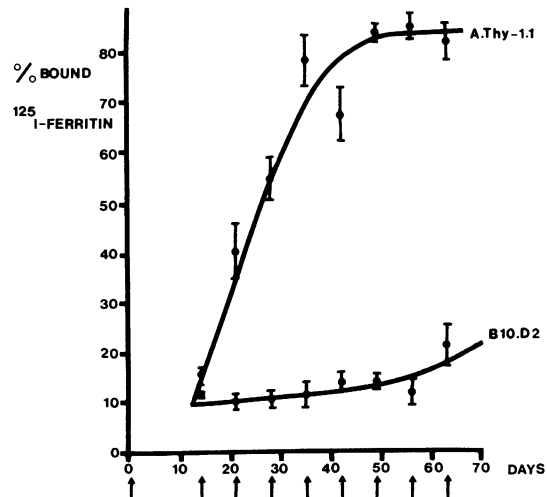


Fig. Immune responses to ferritin in sequentially immunised A.Thy-1.1 and B10.D2 mice after primary immunisation in incomplete Freund's adjuvant. Arrows indicate immunisations.

The immune response to ferritin after primary immunisation in complete Freund's adjuvant and 6 repeated immunisations in PBS, in mouse strains B10.M, A.Thy-1·1, C3H/He, (B10.M × A.Thy-1·1)F1 and (B10.M × C3H/He)F1 hybrids is shown in Table 3. Mouse strains A.Thy-1·1 and C3H/He, which on primary immunisation with antigen in saline are low responders, now become high responders after hyperimmunisation when compared to B10.M mice. Again, it is shown that hyperimmunisation of these mice causes an inversion of the high and low responder status as compared to that obtained after primary immunisation in PBS, and these differences are significant ($P < 0.001$). Furthermore the quantitative immune responses of the (B10.M × A.Thy-1·1)F1 and (B10.M × C3H/He)F1 hybrids after hyperimmunisation is intermediate between that of the parental strains, as indicated by dominance index values close to zero.

ANTIBODY CLASS DISTRIBUTION AFTER HYPERIMMUNISATION

The binding to ferritin by IgG and IgM fractions obtained from B10.D2 and A.Thy-1·1 mice after hyperimmunisations with ferritin is shown in Table 4. It is shown that the low antibody response to ferritin of the B10.D2 mice is not due to a poor conversion of the IgM to IgG response. It is apparent that both strains of mice produce an IgG and IgM response to ferritin. The ratio of the IgG to IgM response to ferritin is approximately 3:1 in both B10.D2 and A.Thy-1·1 mice. Thus the difference in responsiveness to ferritin in these two strains of mice is not due to differences in conversion of IgM to IgG, but is due to the total amounts of antibody of both the IgM and IgG classes of immunoglobulins.

Discussion

It has previously been shown (Young *et al.*, 1976, 1977) that after primary immunisation with ferritin in PBS, A.Thy-1·1 and C3H/He are low responders (Table 1) while B10.M and B10.D2 mice are high responders. Tables 2 and 3 show that inversion of

Table 4 Binding to ferritin by IgG and IgM fractions obtained from B10.D2 and A.Thy-1·1 mice after 4 immunisations of 200 µg ferritin

Strain	Immunoglobulin class	Mean percentage bound of 110 µg ¹²⁵ I-ferritin, by 15 µl of immunoglobulin fraction
B10.D2	IgG	5.35
B10.D2	IgM	2.2
A.Thy-1·1	IgG	37.80
A.Thy-1·1	IgM	12.74

the high and low responder strain status of mice to ferritin occurs after hyperimmunisation with antigen in adjuvant. Thus primary immunisation with antigen in Freund's adjuvant, followed by repeated immunisation with ferritin in saline, causes an inversion of the high and low responder status compared to that obtained after primary immunisation in PBS, in that A.Thy-1·1 mice become high responders and B10.D2 mice low responders. Furthermore the F1 hybrids (B10.M × A.Thy-1·1) and (B10.M × C3H/He) show intermediate responses between that of parental strains, and this is consistent with F1 data obtained from other immunogenetic systems (Ebringer *et al.*, 1976a).

The effects of hyperimmunisation in high and low responder strains to other antigens have been investigated by other workers. Hyperimmunisation of low responder mice to GAT produces no increase in the level of antibodies (Dunham *et al.*, 1972). It has been shown in some immunogenetic systems that for antigens of restricted heterogeneity such as GT, GAT, and GA, an all-or-none type of immune reaction could be expected even after prolonged immunisation (Benacerraf and McDevitt, 1972). Stimpfling *et al.* (1976) have shown that low responder mice hyperimmunised to Ea-2·1 antigen produce no increase in the level of antibodies. Stankus and Leslie (1975) showed that rats producing a low response to streptococcal group A polysaccharide vaccine could not enhance their precipitin response after a secondary series of immunisations.

Conversely it has been reported that high and low responder status of animals can be changed by a

Table 3 Immune response to ferritin after primary immunisation of 200 µg in complete Freund's adjuvant and 6 repeated immunisations in PBS in B10.M, C3H/He, A.Thy-1·1 (B10.M × A.Thy-1·1)F1 and (B10.M × C3H/He)F1 hybrid mice

Strain	Number of mice	Mean (± SE) percentage bound ¹²⁵ I-ferritin by 10 µl undiluted serum	Dominance index
B10.M	31	29.32 ± 1.46	
C3H/He	53	41.94 ± 2.18	
A.Thy-1·1	36	68.87 ± 2.54	
(B10.M × A.Thy-1·1)F1	62	43.89 ± 1.27	- 0.26
(B10.M × C3H/He)F1	59	36.31 ± 1.31	+ 0.36

variety of conditions. Eichmann (1972) found that A/J mice could become high responders to streptococcal polysaccharides if a different immunisation route was used. Low responder mice to IgG (gamma 2a) allotypes, when hyperimmunised, produced moderately raised immune response to this antigen (Liebermann and Humphrey, 1972). It has been shown that a variation in the antigen dose can lead to inversion of high and low responder status to (T,G)-A--L in rats (Koch, 1974), (T,G)-Pro--L in mice (Jormalainen *et al.*, 1975), and streptococcal antigens in humans (Greenberg *et al.*, 1975). The reasons for inversion of the high and low responder status of animals in some immunogenetic systems, and not in others, is at present unknown.

It is known that in some immunogenetic systems the major difference in the responses of high and low lines lies in the class of antibody which appears after multiple injections of antigen (Silver *et al.*, 1972). In some systems low responder animals have a defect in the ability to switch from IgM to IgG production after secondary immunisation (Benedict *et al.*, 1975). This possibility however does not appear to be the cause for inversion in B10.D2 and A.Thy-1.1 mice because it was found that in both strains the ratio of IgG to IgM immunoglobulins to ferritin was 3:1.

It is possible that the immunisation of ferritin in Freund's adjuvant enhances its immunogenicity to a greater extent in some strains of mice than in others and it thus produces an inversion of the immune response. Incomplete Freund's adjuvant enhances the immunogenicity of some natural antigens, such as keyhole limpet haemocyanin, sometimes up to 1000-fold (Dixon *et al.*, 1966). It is unlikely that the mycobacterium component of complete Freund's adjuvant (Luderer *et al.*, 1976) was involved in inversion because the same effect was also observed with incomplete Freund's adjuvant.

An alternative explanation of immune response inversion is provided by the one-gene theory of cross-tolerance hypothesis. If ferritin cross reacts with low responder self antigens, such as those found in A.Thy-1.1 mice (Deacon and Ebringer, 1977), then initially a poor immune response will be produced because few T cells will co-operate with B cells to produce an adequate antibody response. In high responder animals it is suggested the antibodies produced will rapidly opsonise the antigen and lead to its removal by the neutrophils of the reticuloendothelial system. However, in low responder animals any antibodies produced will also have autoimmune activity, thus preventing adequate opsonisation and phagocytosis of the external antigen. With a proliferating micro-organism, the quantities of antigen released would increase and

stimulate many B cell clones to differentiate into plasma cells, thereby producing immune response inversion despite inadequate T cell co-operation. Low responder animals may thus be susceptible to prolonged infection by micro-organisms which cannot be readily eliminated because of partial cross reactivity to self antigens, thereby deviating antibodies towards some autoimmune inflammatory activity with possible pathological sequelae.

The cross-tolerance model thus proposes that susceptible individuals, carrying defined genetic markers, will produce small antibody responses in the early part of an infection. However, in the latter part of the infective process such individuals would produce high levels of antimicrobial antibodies, having some cross-reactive autoimmune specificity which could lead to tissue damage through complement activation thereby releasing altered self antigens and hence producing a self-sustaining chronic inflammatory process.

An example of such a process appears to occur in rheumatic fever. For instance, blood group A individuals have a slightly increased incidence of rheumatic fever compared to blood group O individuals, but so far the mechanism underlying this difference remains obscure (Glynn, 1975).

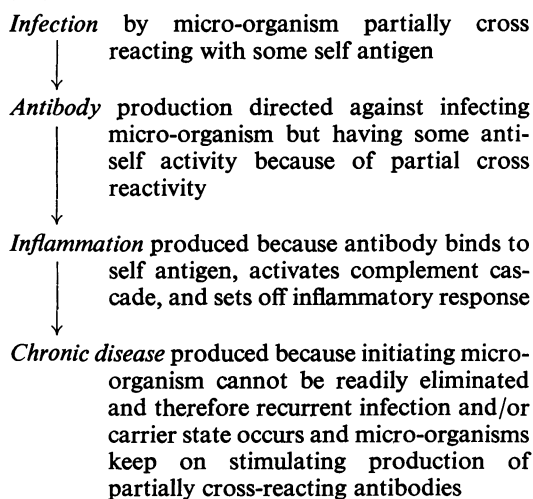
A possible explanation is suggested by the cross-tolerance hypothesis. The terminal sugar residue in the micro-organism causing rheumatic fever, namely *Streptococcus pyogenes* is N-acetyl-(D)-glucosamine, while the terminal residue in blood group A is N-acetyl-(D)-galactosamine and in blood group O it is (L)-fucose. N-acetyl-(D)-galactosamine differs at every carbon atom from (L)-fucose but is identical to N-acetyl-(D)-glucosamine found in streptococcus, except for the position of the hydroxyl group on the fourth C4 carbon atom. Therefore other things being equal, individuals with blood group A are closer to streptococci than those with blood group O and this could be a reason for the increased incidence of rheumatic fever in blood group A individuals (Ebringer *et al.*, 1976b).

Furthermore, subjects secreting Lewis A salivary substance have a higher incidence of rheumatic fever than subjects secreting Lewis B salivary substance and this locus segregates independently from the blood group locus (Glynn and Holborow, 1969). The terminal residue in Lewis A substance is galactose while the terminal residue in Lewis B substance is fucose and therefore the same argument of cross reactivity could apply to this locus as that described for blood groups.

In following antistreptococcal antibodies in patients with tonsillitis, in an American study some years ago (Rothbard *et al.*, 1948) it was found that those individuals who eventually developed rheu-

matic fever tended to have low or delayed antibody responses in the early part of the disease compared to the nonrheumatic group. A similar observation was made by a Scandinavian group of workers, who noted that the antistreptolysin titre curve in rheumatic fever is both more gentle and more prolonged than is the case in uncomplicated inflammation of the throat (Winblad *et al.*, 1949). Although these observations do not establish immune inversion in rheumatic fever, that is a low response in the early phase and a high response in the latter phase of the disease, it is however consistent with such a possibility.

According to the crosstolerance hypothesis, the pathogenesis of a genetic marker-associated disease can be illustrated by the following sequence of events:



The crosstolerance hypothesis thus provides a mechanism of pathological damage which can be applied to several chronic diseases and implicit to this process is the apparent inversion in the quantitative antibody response to the extrinsic antigen. It is suggested that both the ferritin IR-gene system and rheumatic fever could be considered as examples of such a process.

The application of the crosstolerance hypothesis to ankylosing spondylitis suggests that HLA B27 positive individuals should have a poor or low immune response in the early phase of the disease to some cross-reactive extrinsic antigen. Lymphocytes obtained from HLA B27-positive individuals have been shown to have a poor or low thymidine uptake, compared to non-B27 lymphocytes, when cultured with *Yersinia enterocolitica* (Nikbin *et al.*, 1975), an organism known to be associated with a reactive arthritis indistinguishable from ankylosing spondylitis (Aho *et al.*, 1975). However, the appearance of

high antibody titres in the latter phase of ankylosing spondylitis to some Gram-negative micro-organisms such as *Klebsiella* (Ebringer *et al.*, 1977) or any other antigens, as suggested by the crosstolerance hypothesis and the work reported here, remains the subject of further study.

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