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AUTOIMMUNE DISEASE IN INBRED MICE*

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No one, nowadays, disputes the occurrence of autoimmunity in man. The strong tendency of certain autoantibodies to occur predominantly or exclusively in certain human diseases makes it appear very likely that autoimmune reactions can indeed be important initiating and/or continuing causes of localized or generalized tissue damage.

Nevertheless, such a causative role for autoimmunity has so far been demonstrated directly and unequivocally for but few human diseases, and confidence in ascribing pathogenicity to autoimmune systems is based to no small extent on the convincing results of animal experiments in which a variety of diseases, usually organ-specific, have been apparently directly induced by immunization with autologous or isologous tissues.

Further, although this symposium presents evidence that genetic factors play a part in human autoimmunity, it was recently pointed out by Sir Macfarlane Burnet in his Harben lectures that some of the strongest evidence favouring a role for genetic factors in spontaneous autoimmune disease derives from studies on its transmission in the New Zealand Black (NZB) mouse strain and its hybrids.

The characteristics of this strain are now well known, and the findings of Bielchowsky, Helyer & Howie (1959) and of Burnet & Holmes (1963) and others need only be briefly summarized here. Mice of the NZB strain appear normal at birth, but between 4 and 9 months of age a majority develop a haemolytic anaemia analogous to human autoimmune haemolytic anaemia of warm antibody type. The first abnormality detectable in these mice is that their circulating red cells begin to give positive direct antiglobulin tests and eventually the test becomes positive in virtually 100% of the mice. The haemoglobin and PCV begin to fall, the reticulocyte increase in the blood becomes marked, and splenomegaly develops. In addition, plasma cells become prominent in the spleen and lymph nodes, and also in the lungs, kidneys and thymus. The presence of germinal centres in the thymus has been

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noted but their relationship to intrinsic thymic tissue is disputed (Siegler, 1965). In the kidneys, glomerular lesions, with tubular casts, develop in more than 50% of the females and in about 25% of the males, according to Burnet. Finally, a proportion of the mice develop anti-nuclear autoantibodies; in our hands about 20% (Holborow, Barnes & Tuffrey, 1965).

The results of crossing the NZB strain with other inbred mouse strains show that the autoimmune character of the NZB mouse is expressed in its F_1 hybrids, although manifested in different ways. Bielchowsky & Bielchowsky (1964) found that haemolytic anaemia developed in 100% of F_1 hybrids from NZB × NZC matings, independently of the sex of the NZB parent. Howie & Helyer (1965) also found in seven different crosses of NZB with other strains that the F_1 hybrids inherited disease irrespective of the sex of the NZB parent. This indicates that the transmission of disease to the offspring is not sex-linked, and that a milk factor is not involved. It also seems to make transmission of a viral agent less likely as a causative factor.

Howie & Helyer's study also showed that the character of the principal disease developing in the hybrids is considerably modified by the contribution of the non-autoimmune parent.

Autoimmunity in the NZB mouse is thus an inherited character and is expressed in the heterozygote, but Burnet & Holmes (1965) showed that the action of more than just a single gene must be implicated. They studied the age at which the change from a negative to a positive antiglobulin test occurred in mice produced by crossing NZB mice with NZW, C3H, AKR and C57BL strains, and by back-crossing these F_1 hybrids with either parent, and plotted the age-specific incidences of conversion to a positive antiglobulin test. They found that the differences between the plots for the parent strains, the F_1 hybrids and the backcross progency could not be explained by inheritance of a single dominant gene. Following Burch's theory of human autoimmune disease (Burch, 1963), Burnet & Holmes (1965) suggest that both an appropriate genotype and a process of somatic mutation are necessary for the manifestation of NZB disease, and they propose that the phenotypic expression of the final somatic mutation depends upon the nature of a gene complex involving at least three to five unlinked genes.

The autoimmune abnormality of NZB mice thus appears to be, at least in part, an expression of genetic constitution. Cell transfer experiments carried out by Holmes, Gorrie & Burnet (1961) were designed to show whether the abnormality is transmissible by the lymphoid cells themselves. When suspensions of spleen cells from affected NZB mice were injected intraperitoneally into young NZB mice as yet unaffected, the red cells of the young recipients subsequently developed positive antiglobulin tests. Results of typical transfer experiments in our hands are shown in Table 1. Suspensions of spleen cells from NZB mice about 9 months old were injected intraperitoneally into young recipient NZB mice about 8 weeks old and of the same sex as the donors. Each mouse received cells (approximately 100 million) from only one donor. The recipients were bled at weekly intervals and their red cells tested for antiglobulin reactivity; the packed cell volume was also measured and the blood reticulocytes counted. No positive antiglobulin tests were seen until the 10th day after transfer and all positives had appeared by the 3rd week. By the 6th week all antiglobulin tests had become negative again. No difference was noted between irradiated and non-irradiated recipients.

This sequence of events—a delay of 1–2 weeks, followed by the appearance of antibody

in a number of the recipients and, after 4–6 weeks, the subsequent decline of antibody—is essentially that reported by Holmes *et al.* (1961), although in their experiments some of the recipient mice continued to show positive tests until they reached an age when spontaneous Coombs conversion might have been expected. In our experiments reticulocyte counts were variable, but high levels (20–40%) were found in the positive non-irradiated mice. No consistent changes were observed in the packed cell volume during the period of the experiment.

Two broadly different explanations of these results may be put forward. Either the transferred cells themselves are the source of the antibody found on the host's red cells, or the donor material administered stimulates the host's own lymphoid cells to premature autoantibody production.

If we accept the first of these explanations, that the observed effects are the result of an adoptive immunity, we must conclude that the donor's spleen cells are already primed, that is, have already encountered the relevant tissue antigens in the donor mice. Burnet proposes that the genetic factors discussed above may operate by permitting survival of

TABLE 1. Effect of spleen cell transfer on the antiglobulin reaction

Donor cells (9 month NZB)	Recipients			
	6–8 weeks NZB		6–8 weeks NZW irradiated	6–8 weeks C_3H irradiated
	Non-irradiated	Irradiated		
Whole spleen cell suspension	2/4*	0/4	0/12	0/12
Filtered spleen cell suspension	3/5	2/4	—	—
No cells	0/3	0/3	0/8	0/10

* No. developing + antiglobulin test/No. receiving cells.

cells primed against autoantigens in this fashion. We know from the work of Mäkelä & Mitchison (1965) that primed cells on transfer to a normal or irradiated host require a further antigenic stimulus before producing a secondary response in the latter. If results such as those described here are truly attributable to adoptive immunity, the transferred cells must be receiving such antigenic stimulation in the host, for the delay in the appearance of the antiglobulin test is against passively transferred antibody being responsible for coating the red cells.

Some of the recipients shown in Table 1 were irradiated before receiving the donor cells. This was done for two reasons. The activity of transplanted cells is enhanced in the irradiated recipient (Harris *et al.*, 1954) and, secondly, the recipients' own lymphoid cells are thus rendered unresponsive. The duration of unresponsiveness of NZB mice following different doses of irradiation was separately investigated and results are shown in Table 2. It will be seen that 500 r suppressed the circulating antibody response to weekly injection of mouse red cells for about 21 days. The appearance of positive antiglobulin tests earlier than this in the irradiated recipients suggests that this is not due to a response by the recipients' own lymphoid tissues.

Table 1 also shows that transfer of antiglobulin positivity may be achieved not only with unmodified suspensions of mixed spleen cells, but also with cells obtained by filtering the mixed cell suspension through glass wool according to the method of Hildemann, Linscott & Morlino (1962). The resulting cell suspension, containing >90% small lymphocytes, proved capable of transferring antiglobulin reactivity to both irradiated and intact recipients.

Whether adoptive autoimmunity in NZB mice shows the same pattern as adoptive immunity to foreign antigens was also investigated by following the behaviour of donor cells labelled with tritiated thymidine. The lymphoid tissues of the recipients were examined histologically for labelled blast cells with pyroninophilic cytoplasm. The tissues were taken from recipients killed within 100 hr of irradiation when DNA synthesis was noted to be minimal and re-utilization of donor thymidine, therefore, unlikely. Labelled pyroninophilic large lymphocytes were present in the recipient spleens in considerable numbers.

TABLE 2. Haemagglutinin response to human red cell stroma in irradiated mice

Strain	X-ray dose (r)	1 week	2 weeks	3 weeks
NZB	0	3/4*	4/4	4/4 (64–512)†
	300	2/4	2/4	4/4 (32–64)
	400	0/4	4/4	3/4 (8–32)
	500	0/4	1/4	2/4 (8)
	600	0/4	2/4	2/4 (4–8)
C_3H	500	0/4	2/4	1/4 (8)

* No. in each group of four showing haemagglutinin response.
† Range of haemagglutinin titres at 3 weeks.

In accepting adoptive immunity as an explanation for the results of cell transfer in NZB mice, we have assumed that the stimulus received by the transplanted cells originates in the host animal. Alternatively, however, the antigen might be carried in from the donor with the primed cells, and subsequent loss of this passively-acquired antigen might account for decline in antibody production in the recipients.

In recent experiments irradiated NZW or C_3H mice receiving spleen cells from older NZB mice have not developed positive antiglobulin tests (Table 1). This is not due to premature rejection of the transferred cells, for irradiated recipients receiving similar cells from NZB mice immunized against sheep cells gave a good haemagglutinin response on challenge with sheep cells. In these circumstances, the persistent negativity of the antiglobulin tests implies that, in other strains, transplanted NZB cells receive no appropriate antigenic stimulus. Successful intra-strain transfer of antiglobulin positivity from old to young NZB mice thus appears to depend upon something more than contact between transferred cells and normal tissue antigens in the recipient. It will be interesting to see whether spleen cells from young NZB mice are stimulated to autoantibody production on transfer to old irradiated NZB recipients but we have not yet evaluated this situation.

In any event, we have to consider the possibility that NZB mice may not only produce lymphoid cells predisposed to autoimmune reactivity, but may also possess or harbour antigens especially effective in eliciting autoimmune responses.

The cell transfer experiments we have described here are mainly exploratory in nature, but they give results that accord well with those described by Burnet & Holmes (1963, 1965). They also show that adoptive immunity provides a versatile experimental method suitable for investigating further the spontaneous autoimmunity of NZB, and perhaps capable of distinguishing the relative importance of genetic and environmental factors in producing it.

GENERAL DISCUSSION

DR LEONHARDT. Hereditary hypergammaglobulinaemia in mink or Aleutian disease has been shown to be due to a filterable agent, probably a virus infection. Can you be sure that the autoimmune disease in the NZB mice is not due to an outside agent such as a virus?

DR HOLBOROW. No. I don't think this can be excluded. We have just found that germ-free NZB mice develop autoimmune disease (East & Holborow, unpublished observations). This does not, of course, exclude a viral infection.

DR DONIACH. Your experiments fit in with Dr Mellor's finding that immunoglobulin deposits eluted from NZB/B1 kidneys did not react with the glomeruli of normal mice but could be attached to other NZB nephritic kidneys (Mellers, 1965).

DR MACDONALD. In the established case of autoimmune haemolytic anaemia in the NZB mice are all the red cells Coombs positive and how long does a Coombs positive red-cell survive in these mice?

DR HOLBOROW. We ourselves have not made observations on this. Barnes & Tuffrey (1966) have done some work on this and have found that the Coombs-positive cells are more quickly destroyed.

PROFESSOR CLARKE. Have you tried to protect hybrid NZB mice 'at risk' for autoimmune nephritis (Russell, Hicks & Burnet, 1966) by giving passive antibody obtained from mice which have the developed disease? This idea stems from our Rhesus work, where we have shown that it is possible to protect 'at risk' Rhesus negative women by giving anti-D γ-globulin (Finn, *et al.*, 1961).

DR HOLBOROW. No, we have not tried that.

DR GOUDIE. Could you tell us the incidence of Coombs tests in the F_1 hybrid between NZB and NZW mice?

DR HOLBOROW. Burnet & Holmes (1965) report that in the NZB/NZW hybrid the Coombs test is only rarely positive and then only in old age and in females; the male mice die before they become Coombs positive.

DR GOUDIE. This is interesting since these red cells must carry the antigen of the NZB which seems to be of importance.

DR HOLBOROW. The red cell antigen may react with, but may not be stimulating, the production of antibody.

DR GOLDBERG. What is the effect of corticosteroid therapy on the development of haemolytic anaemia in these mice?

DR HOLBOROW. Giltman, Holmes & Burnet (1965) have studied this and can prevent the onset of haemolytic anaemia by using corticosteroids.

DR IRVINE. It would be appropriate to consider the thymus changes in human autoimmune disease. In several diseases associated with autoimmunity there are thymic abnormalities including the occurrence of germinal centres, alteration in the number of plasma cells, spindle epithelial

cells and cystic Hassall's corpuscles (Gunn, Michie & Irvine, 1964; Irvine & Sumerling, 1965; Mackay, 1966). These thymic changes provide a link between the autoimmune diseases in man and the occurrence of an autoantibody type of haemolytic anaemia and of lupus nephritis in the NZB strain and NZB × NZW hybrid mice. Of particular interest is the different histopathology of the thymus in patients with organ-specific autoimmunity, e.g. thyroid disease and in patients with non-organ-specific autoimmunity, e.g. systemic lupus erythematosus. While it is possible that the thymic abnormalities are a consequence of an autoimmune process, it is also conceivable that the function of the thymus as a controller of immunological homeostasis has been lost and that forbidden clones of immunologically competent cells are no longer eliminated as in the normal individual or animal. The precise nature of the defect in immunological homeostasis is not understood, but it would appear to be genetically determined and it is conceivable that it is mediated through the thymus (Irvine, 1964).

DR MACSWEEN. We have had the opportunity of studying the thymus from three patients with systemic lupus erythematosus and two patients with rheumatoid arthritis, in whom the thymus was removed as a therapeutic measure (MacSween, Anderson & Milne, 1967; Milne *et al.*, 1967). In all three patients with systemic lupus erythematosus some degree of involution was evident, but contrary to the observations of Burnet & Mackay (1965) and Hutchins & Harvey (1964), some cortical remnants persisted in the three glands. Hyperplastic changes with active germinal centres were seen in the three glands and it was of interest that in the only patient who had not received corticosteroid treatment pre-operatively the degree of hyperplasia was less than in one of the patients of the same age who had received steroid therapy for 6 months prior to operation. In the two patients with rheumatoid arthritis the thymus was markedly atrophic in one, but the other glands showed medullary hyperplasia with numerous large germinal centres and again this patient had been on large doses of steroids for some years preoperatively.

DR ROWELL. Was there any change in these patients after thymectomy?

DR MACSWEEN. No, they are essentially unchanged clinically.

PROFESSOR ANDERSON. The development of thymic germinal centres in patients with various autoimmune diseases provides strong evidence that an immune response is occurring in the thymus; there are two main theories on its nature. Firstly, that the thymus is reacting immunologically against autoantigens present in other organs. Secondly, that the changes represent an 'autoimmune thymitis', i.e. that the germinal centres represent an autoimmune response to thymic antigens, just as the lymphoid infiltration in the thyroid in Hashimoto's disease is thought to represent an autoimmune thyroiditis. I would like to ask Dr Holborow if he found large numbers of mast cells in the thymus of his NZB mice, as described by Burnet?

DR HOLBOROW. Yes, you see mast cells in the thymus in the older mice.

DR DENMAN. There are two points which are relevant. First, if steroids are given to mice for long periods, in the early stages the lymphocytes are destroyed but after a few weeks the lymphocytes become resistant and the thymus then returns to its normal size. Secondly, Siegler (1965) has reported the occurrence of thymic lymphoid follicles in aged Swiss mice in the absence of haemolytic anaemia.

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