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Development of Coombs Positive Haemolytic Anaemia in NZB Mice Injected with Freund's Complete Adjuvant

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Summary. Intraperitoneal injection of young adult NZB mice with Freund's complete adjuvant emulsion, alone or incorporating BALB/c mouse erythrocytes, resulted in accelerated direct Coombs conversion and development of splenomegaly. Incorporation of adjuvant with NZB erythrocytes caused an accelerated reactivity in some animals, but in others resulted in a delayed Coombs conversion. In contrast, positive Coombs tests failed to develop at all in NZB mice injected with a Freund's complete adjuvant preparation containing ten times the usual amount of *Mycobacterium*. These observations might be explained in part by the relative influences of adjuvancy and of antigenic competition on the availability of precursor cells capable of responding to autoantigenic stimulation.

INTRODUCTION

Young adult NZB mice have been observed to be immunologically hyperactive. East, de Sousa and Parrott (1965) and de Vries and Hijmans (1967) have noted marked lymphoid hyperplasia and abnormal proliferation of plasma cells to develop in these animals well before the onset of autoimmune disease. NZB mice have been observed also to be hyper-responsive to stimuli by foreign antigens (Morton, Olson and Siegel, 1967; Weir, McBride and Naysmith, 1968; Morton and Siegel, 1969; Staples and Talal, 1969a), to show a relatively early development of immune competence (Evans, Williamson and Irvine, 1968; Playfair, 1968) and to resist experimental induction of immunological tolerance (Staples and Talal, 1969a, b). A 100-fold greater dose of sheep erythrocytes has been shown necessary to saturate the spleen plaque-forming capacity of NZB as compared to BALB/c strain mice (Morton and Siegel, 1969). These observations have led to the speculation from this laboratory (Morton *et al.*, 1967; Morton and Siegel, 1969) that the development of autoimmune disease in NZB mice may be due to the presence of an unusually large pluripotential stem cell pool with a concomitantly augmented opportunity for the triggering of immune responses that may result in the formation of autoantibody.

The successful experimental induction of autoimmune disease in laboratory rodents has been achieved mainly through incorporation of tissues in complete Freund's adjuvant (Rose and Taylor, 1965). Work from this (Siegel and Morton, 1966, 1967; Morton, Gaglia, Wilkins and Siegel, 1968) and other laboratories (Old, Clarke, Benacerraf and Goldsmith, 1960; Munoz, 1964) has suggested that one function of adjuvant materials might be to increase the number of haemopoietic stem cells. Conceivably, then, a similar basic mechanism could be operative in both the development of autoimmune disease in

NZB mice and the production of adjuvant-autoimmunity. In this connection, experiments were undertaken to determine whether the development of Coombs positive haemolytic anaemia in NZB mice could be accelerated by the injection of Freund's complete adjuvant emulsion, either alone or with incorporated syngeneic NZB or allogeneic BALB/c red blood cells.

MATERIALS AND METHODS

Mice

The NZB mice used as breeding stock were kindly provided as generations 57 and 58 by Mr W. Hall, University of Otago Medical School, Dunedin, New Zealand. Animals of both sexes from the third and fourth generations resulting from brother–sister matings in this laboratory were used in the present investigations. For comparative studies, male and female BALB/c mice obtained from Jackson Laboratory, Bar Harbor, Maine, were employed.

Adjuvant preparations

Three different adjuvant systems were employed in three separate experiments. In the first study, eight 8-week-old male and female mice of the BALB/c strain provided the BALB/c erythrocytes, while the NZB erythrocyte donors consisted of eight 4-month-old Coombs negative male and female animals. Donor mouse blood cells were collected in Alsever's anti-coagulant and washed three times in isotonic saline, care being taken to remove the buffy coat. One volume of packed red cells was then emulsified with 0·5 volume of saline and 1·5 volumes of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan). Freund's complete adjuvant (CFA) was also prepared by emulsion of the Difco adjuvant preparation with 1 volume of isotonic saline in the absence of erythrocytes. In a second study, 2 volumes of Difco Freund's complete adjuvant were emulsified with 3 volumes of isotonic saline. This preparation and those used in the first study are referred to in the text as low dose mycobacterial adjuvants. In the third study, as high dose mycobacterial adjuvant, 25 mg *Mycobacterium butyricum* (Difco Bacto M. Butyr. desiccated, control 513026) was incorporated into 2·5 ml Freund's incomplete adjuvant (Difco) and emulsified with 2·5 ml saline. In each experiment, recipient animals were injected intraperitoneally with 0·25-ml volumes of the respective emulsions.

Haematology

Peripheral blood for haematological determinations was obtained by bleeding from the retro-orbital plexus. Total counts of nucleated cells were carried out using a model A Coulter electronic cell counter, and differentials were performed on blood smears stained by a modified Wright–Giemsa method (Reich, 1954). Individual 60 lambda blood samples were collected in heparinized hematocrit tubes for direct Coombs assays (Norins and Holmes, 1964).

RESULTS

Experiment I. Effect on NZB autoimmune disease of Freund's complete adjuvant incorporating syngeneic and allogeneic erythrocytes

Three-month-old NZB mice were divided into four groups, each receiving a single intraperitoneal injection of either: (1) NZB erythrocytes in Freund's complete adjuvant

(NZB-RBC in CFA), (2) BALB/c erythrocytes in Freund's complete adjuvant (BALB/c-RBC in CFA), (3) Freund's complete adjuvant–saline emulsion without incorporated erythrocytes (CFA) only, or (4) saline alone. Transient wasting and diarrhoea, previously observed in the case of BALB/c mice injected with Freund's complete adjuvant (Morton and Siegel, 1966), were not seen to develop in adjuvant-injected NZB mice. As noted in Table 1, accelerated development of Coombs reactivity occurred in the mice receiving

TABLE 1

DEVELOPMENT OF COOMBS REACTIVITY WITH TIME IN NZB MICE FOLLOWING A SINGLE INTRAPERITONEAL INJECTION OF FREUND'S COMPLETE ADJUVANT (CFA) WITH AND WITHOUT INCORPORATED NZB OR BALB/c ERYTHROCYTES

Treatment	Days after adjuvant injection					
	14	30	57	82	111	138
Saline	0/6	0/6	0/6	1/6	3/6	6/6
CFA only	0/6	0/6	0/6	3/6	5/6	6/6
CFA+NZB-RBC	0/8	0/8	1/8	2/8	2/7	2/7
CFA+BALB/c-RBC	0/8	1/8	4/8	4/8	6/8	8/8

Values are reported as ratios of animals showing Coombs conversion in a given treatment group.

Freund's complete adjuvant alone and in mice receiving BALB/c-RBC in CFA. In animals receiving NZB-RBC in CFA an acceleration was noted in the case of two animals, a majority of the mice manifesting delay in the Coombs conversion. By the age of 10½ months all mice were Coombs positive; and, upon sacrifice, a number of animals in each of the adjuvant-treated groups displayed marked splenomegaly as compared to the saline injected controls (Table 2).

TABLE 2

INDIVIDUAL SPLEEN WEIGHTS OF MICE (TABLE 1) RECEIVING VARIOUS ADJUVANT TREATMENTS AND KILLED AT 10½ MONTHS OF AGE

Treatment	Spleen weights (mg)						
Saline	166,	176,	234,	246,	268,	282	
CFA only	150,	328,	346,	462,	543,	641	
CFA+NZB+RBC	176,	241,	323,	501,	631,	679	
CFA+BALB/c-RBC	164,	193,	267,	305,	441,	983,	1909

EXPERIMENT II. EFFECTS OF INJECTION OF ONE AND OF THREE DOSES OF FREUND'S COMPLETE ADJUVANT–SALINE EMULSION WITHOUT INCORPORATED ERYTHROCYTES

The following experiment was carried out to confirm and extend the previous finding that Freund's complete adjuvant alone might have an accelerative effect on the development of the Coombs positive autoimmune disease state. One group of 45-day-old male and female NZB mice was given a single injection of Freund's complete adjuvant–saline emulsion, while a second group received a series of three adjuvant injections at 45, 78 and 128 days of age. A third group of NZB mice received three similarly spaced injections of isotonic saline. Groups composed of three male and three female BALB/c mice of comparable ages were likewise studied under each of these experimental conditions. Profound wasting and diarrhoea were seen to develop in the BALB/c mice after each adjuvant injection, while no such effects were observed in the case of similarly treated NZB mice. By 6 months of age all of the female NZB mice which had received three injections of

Freund's complete adjuvant developed abdomens distended with ascitic fluid. This condition was not observed to develop in any of the other groups of mice included in this study. As seen in Table 3, NZB mice in the two adjuvant-treated groups demonstrated earlier development of Coombs reactivity than did the untreated NZB mice. The multiple injection regimen of CFA was productive of earlier acceleration as compared to the single

TABLE 3

DEVELOPMENT OF COOMBS REACTIVITY IN MICE FOLLOWING ONE OR THREE INTRAPERITONEAL INJECTIONS OF FREUND'S COMPLETE ADJUVANT (CFA) COMPARED WITH MICE RECEIVING SALINE ONLY

Treatment	No. mice	Age (months)	
		6	9
NZB			
Saline (×3)	19	0/19	5/19
CFA (×1)	25	1/25	12/20
CFA (×3)	19	3/19	9/15
BALB/c			
Saline (×3)	6	0/6	0/6
CFA (×1)	6	0/6	0/6
CFA (×3)	6	0/6	0/6

Results are expressed as numbers of mice Coombs positive/total mice in treatment group.

CFA injection, but by the age of 9 months the two groups displayed identical percentages of Coombs positivity. As noted in Table 3, corresponding BALB/c mice failed to manifest positive Coombs tests over the entire period studied.

EXPERIMENT III: EFFECT OF HIGH DOSE MYCOBACTERIAL ADJUVANT ON NZB AUTOIMMUNE DEVELOPMENT

A further experiment was undertaken to study the effect on autoimmune acceleration of incorporating a larger amount of *M. butyricum* in the adjuvant mixture. Here, fourteen 3-month-old male and female NZB mice were injected intraperitoneally with an adjuvant emulsion containing 1·25 mg *M. butyricum.* Eight untreated NZB mice of the same age were followed as controls. Although no wasting was noted following adjuvant injection, the treatment was observed to be highly toxic, one-half of the mice dying during the 1st month (Table 4). By 13 days post-adjuvant administration all of the mice showed abdomens distended with ascitic fluid, which persisted until death. Organs of adjuvant-treated mice were found, upon autopsy, to be fused by massive abdominal adhesions.

Thirteen days after adjuvant injection a marked increase in peripheral blood nucleated cell counts, characterized by an inverted lymphocyte/granulocyte ratio, was observed (Table 4). Absolute numbers of lymphocytes of CFA-treated mice remained normal or were somewhat depressed at this time, averaging 2430 cells/mm^3 as compared to an average lymphocyte count of 3690/mm^3 for the control of NZB mice. Blood smears from adjuvant-injected mice demonstrated an abundance of abnormal red cell forms: basophilic erythrocytes many of which had a frayed appearance, and bizarre immature red cell precursors, including stippled cells with degenerate nuclei, were prominent. Nucleated cell counts were essentially normal by 32 days and remained so thereafter, while the lymphocyte–granulocyte ratio at 32 days was still reversed. Haematocrits became markedly depressed

TABLE 4

SURVIVAL AND HAEMATOLOGY OF NZB MICE INJECTED WITH ADJUVANT CONTAINING A LARGE DOSE OF *M. butyricum* (CFA + 1·25 mg *M. butyricum*) AND OF CONTROL UNTREATED NZB MICE

	Treatment	Days after adjuvant injection			
		13	32	180	240
Survivals	CFA + 1·25 mg *M. butyricum*	13/14	7/14	6/14	4/14
	Controls	8/8	8/8	8/8	8/8
Average nucleated cells/mm³ (range)	CFA + 1·25 mg *M. butyricum*	17,900 (9,100–35,000)	5,043 (3,300–7,100)	7,800 (6,100–10,600)	–*
	Controls	5,050 (4,100–5,800)	3,962 (2,500–5,200)	5,987 (4,100–9,000)	
Average lymphocytes/granulocytes	CFA + 1·25 mg *M. butyricum*	14/86	27/73	–	–
	Controls	76/24	73/27		
Average haematocrits (range)	CFA + 1·25 mg *M. butyricum*	34(30–39)	38(33–42)	35(19–48)	33(26–37)
	Controls	53(49–56)	48(43–52)	46(43–49)	41(37–43)
Coombs positive/total mice	CFA + 1·25 mg *M. butyricum*	0/13	0/7	0/6	0/4
	Controls	0/8	0/8	0/8	5/8

*Not done.

following adjuvant injection, these low values subsequently persisting throughout the experiment. Control mice showed normal haematocrit values until day 240; at this time most of the mice were anaemic and positive Coombs reactivity was evident in five of the eight animals. In the case of the high dose mycobacterial adjuvant-treated mice, Coombs reactivity was not observed to develop at any time during the period of study.

DISCUSSION

Intraperitoneal injection of young adult NZB mice with Freund's complete adjuvant emulsion (CFA) or with BALB/c erythrocytes incorporated into CFA was observed to result in accelerated Coombs conversion. The injection of NZB erythrocytes in CFA resulted in a delayed Coombs conversion in some of the animals, an observation for which we at present have no clear explanation. It should be noted, however, that these animals, as well as the other experimental and control NZB mice, were all Coombs positive when killed at 10½ months of age. At this time, five out of six mice receiving CFA alone showed spleen weights exceeding the highest spleen weight value observed for mice in the saline-injected control group, as did four out of six mice from the NZB-RBC in CFA group and four out of seven mice from the BALB/c-RBC in CFA group. This augmented splenomegaly could be attributed in part to the stimulating effect of adjuvant on the reticuloendothelial system (Morton and Siegel, 1966) in addition to the expansion of splenic red pulp by extramedullary haematopoietic tissue associated with the antibody-induced anaemia (East *et al.*, 1965). Two unusually large spleens (0·98 and 1·91 g) were noted in mice receiving BALB/c-RBC in CFA; this group of animals also manifesting the greatest acceleration of Coombs positive development (Tables 1 and 2). In this regard, the relatively more marked acceleration of autoimmune disease by adjuvant incorporating BALB/c-RBC could conceivably have been a consequence of formation of cross-reacting anti-erythrocyte antibodies.

The accelerative effect achieved by intraperitoneal injection of mycobacterial adjuvant without additionally incorporated antigen might suggest that systemic changes, such as qualitative or quantitative alterations in host cell populations, may have contributed to the earlier formation of autoreactive antibodies. The possibility that antibody formed to *M. butyricum* would cross react with mouse erythrocytes and thus account for accelerated Coombs conversion seems rather unlikely. In the present experiments, for example, BALB/c mice injected with CFA were not observed to develop Coombs positive reactivity. Thompson, Crawford and Severson (1969), likewise, did not observe positive Coombs tests in C57BL/6 mice injected with adjuvant or adjuvant containing syngeneic or allogeneic erythrocytes. Further, administration of adjuvant containing 1·25 mg *M. butyricum*/dose, approximately ten times the amount contained in the usual CFA preparation, appeared to interfere with the development of Coombs conversion. This latter failure to develop positive Coombs reactivity may have been the consequence of a competition-of-antigens effect exerted by the large dose of mycobacterium. In this connection, Isakovic and Waksman (1965) observed that the incidence of adjuvant arthritis in rats, a probable autoimmune manifestation, was diminished when bovine serum albumin was incorporated into the Freund's complete adjuvant. More recently, Pearson and Wood (1969) have reported a reduced induction of adjuvant arthritis when 10 mg ovalbumin (HEA), bovine γ-globulin or rat γ-globulin was incorporated into a mycobacterial wax D adjuvant emulsion. This effect was found to be dose dependent, the incidence of arthritis being

reduced from 95 per cent for the controls to 18·5 per cent for rats receiving 10 mg HEA and to 37·5 per cent for rats receiving 5 mg HEA, while lower dosages were not protective.

Alternatively, competition might be initiated by injection of high dose mycobacterial adjuvant, not as a result of the immunogenicity of the mycobacterium itself, but as a consequence of aberrations induced in haemopoiesis (Table 4; Sri Ram, DeLellis and Glenner, 1969). In this situation, conceivably, abnormal proliferative pressures might result in the diversion along other haemopoietic pathways of cells which would otherwise be available for autoantibody formation. A similar leucocytosis and granulocytosis with accompanying lymphopenia, noted here with NZB mice after administration of high dose mycobacterial adjuvant, has been observed previously with BALB/c mice following a single injection of bovine serum albumin (BSA) in low dose mycobacterial adjuvant (Morton and Siegel, 1966). In the case of the BALB/c animals, these haematological changes were observed to parallel the inductive and productive phases of anti-BSA antibody formation. The haematocrit depression and erythrocyte abnormalities obtaining with high dose mycobacterial adjuvant were not observed, however, in either the BALB/c or NZB mouse strains injected with the low dose adjuvant.

In experiments designed to produce large quantities of antibody in mice, ascitic fluid production has been obtained by several intraperitoneal injections at 4–7-day intervals of Freund's complete adjuvant (Anacker and Munoz, 1961; Jordan, Banovitz, Trapani and Campbell, 1961) or of *Staphyloccocus aureus* cells in Freund's incomplete adjuvant (Lieberman, Mantel, Humphrey and Blakely, 1962). Development of ascites in the present studies was observed to be dosage dependent, appearing by 13 days in high dose mycobacterial adjuvant-treated NZB mice of both sexes and only after 4½ months in female NZB mice receiving three widely-spaced injections of low dose mycobacterial adjuvant.

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