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ACCELERATION OF AUTOIMMUNITY IN NZB/NZW F1 MICE BY GRAFT-VERSUS-HOST DISEASE

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SUMMARY

Chronic graft-versus-host (GVH) disease was induced in NZB/NZW F1 (B/W) hybrid female mice by the weekly injection of parental NZB spleen cells. Control mice received injections of syngeneic spleen cells only. The mice were assayed for antibodies to [^{3}H]DNA and [^{3}H]polyadenylic–polyuridylic acid by a cellulose ester filter radioimmunoassay, and for antibody to thymocytes by a cytotoxicity method.

GVH disease accelerated the development of all three autoantibodies in B/W mice. In addition, sucrose density gradient ultracentrifugation of pooled sera suggested that an accelerated switch from 19S to 7S anti-DNA production may be an early effect of GVH. The mechanism of acceleration is discussed in terms of immunological and viral factors generated by the GVH reaction.

INTRODUCTION

Chronic graft-versus-host (GVH) disease of mice is associated with the development of immunohaemolytic anaemia (Oliner, Schwartz & Dameshek, 1961), glomerulonephritis (Lewis *et al.*, 1968) and lymphoma (Schwartz & Beldotti, 1965; Armstrong *et al.*, 1970). New Zealand Black (NZB) and NZB/NZW F1 hybrid (B/W) mice spontaneously develop a disease most closely resembling human systemic lupus erythematosus (SLE) but similar in certain respects to GVH disease. They develop a Coombs' positive haemolytic anaemia (Bielschowsky, Helyer & Howie, 1959), an immune complex glomerulonephritis (Lambert & Dixon, 1968; Dixon, Oldstone & Tonietti, 1971), B-cell malignancies (Mellors, 1966; Greenspan *et al.*, 1974), and monoclonal macroglobulinemia (Sugai, Pillarisetty & Talal, 1973). In addition, antibodies to DNA (Steinberg, Pincus & Talal, 1969) and RNA (Talal, Steinberg & Daley, 1971; Talal *et al.*, 1971) spontaneously develop in these mice. A naturally occurring thymocytotoxic autoantibody occurs in both NZB and B/W mice, although earlier and in higher titre in NZB mice (Shirai & Mellors, 1971, 1972). We have found that anti-

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nucleic acid antibodies and anti-thymocyte antibodies are separate and independent expressions of autoimmunity with neither activity dependent upon the other (Goldblum, Pillarisetty & Talal, 1975).

Normal strains of mice that ordinarily do not produce autoantibodies may produce antinuclear factor under the influence of GVH disease (Fialkow, Gilchrist & Allison, 1973). Accordingly, we tested the effect of chronic GVH disease on autoantibody production in B/W mice. Our results suggest that GVH disease accelerates the production of antibodies to DNA, RNA and thymocytes in a strain of mice predestined to develop autoimmunity. These results are discussed in terms of possible immunological and viral mechanisms responsible for the pathogenesis of New Zealand mouse disease (Talal, 1970).

MATERIALS AND METHODS

Mice

NZB and B/W mice were from our colonies maintained at the Vivarium of the University of California, San Francisco and the Fort Miley Veterans Administration Hospital. C57Bl/6 mice were purchased from Jackson Laboratory, Bar Harbor, Maine.

GVH protocol

Spleen cell suspensions were prepared by gentle mincing on sterile stainless steel mesh. The cells were washed in cold sterile RPMI 1640 medium (Pacific Biological Company, Berkeley, California). Ten $4\frac{1}{2}$-month-old recipient female B/W mice were given thirteen intraperitoneal injections of 10^7 donor parental NZB spleen cells at weekly intervals (GVH group). Nine age- and sex-matched B/W mice were injected with 10^7 syngeneic B/W spleen cells at identical intervals (control group). Donor spleen cell suspensions came from animals 2–16 weeks old. All mice were bled by orbital sinus puncture prior to the initial injection and at monthly intervals thereafter.

Anti-RNA and anti-DNA assays

Antibodies to RNA and DNA were measured by a cellulose ester filter radioimmunoassay method (Attias, Sylvester & Talal, 1973). These are essentially different antibody populations (Talal & Gallo, 1972). Serum (10 μl of undiluted serum in anti-RNA assay, 20 μl of serum diluted 1/16 in anti-DNA assay) was diluted with borate sodium chloride buffer (0·15 M pH 8·0) and incubated at 56°C for 30 min to destroy complement components which bind some nucleic acids non-specifically. Five microlitres of [^{3}H]polyadenylic–polyuridylic acid (rA–rU) (specific radioactivity 11·6 μg/Ci, from Miles Laboratories, Incorporated, Elkhart, Indiana) was added in the anti-RNA assays; 10 μl of [^{3}H]KB DNA (specific radioactivity 4·3 μg/μCi, from Electronucleonics Laboratories, Incorporated, Bethesda, Maryland) was added in the anti-DNA assays. Final volume of individual assays was always 100 μl. Incubations were carried out for 30 min at 37°C followed by overnight incubation at 4°C. The antigen–antibody mixture was then diluted with buffer and passed over a cellulose ester filter under suction. The filter was washed with buffer, dried, placed in counting vials and covered with 10 ml of Liquifluor–toluene scintillation medium. Radioactivity was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3380, Packard Instrument Company, Downer's Grove, Illinois). The results are expressed

as counts per minute (ct/min) retained on the filter. The retained radioactivity is directly related to serum antibody concentration (Talal & Gallo, 1972).

Cytotoxicity assay for antibodies to thymocytes

Assays for antibodies to thymocytes were performed by a previously described cytotoxicity method (Shirai & Mellors, 1971). A suspension of thymocytes from C57Bl/6 mice age 6–10 weeks was washed three times in Eagles medium (MEM) supplemented with 3% foetal calf serum (heat-inactivated) and adjusted to a concentration of 10^7 cells/ml. Twenty-five microlitres of the cell suspension were then incubated at 4°C for 60 min with 50 μl of the appropriately diluted serum to be tested. The cells were then washed twice and incubated for 30 min at 37°C with 50 μl of rabbit complement at 1/20 dilution (Hyland Laboratories, Costa Mesa, California). The complement was selected for low toxicity towards thymocytes and high complement activity. Cytotoxicity was determined by Trypan Blue dye exclusion.

A control consisting of only thymocytes and complement (no serum) was performed with every set of determinations. Known positive and negative controls were included with each set of tests.

Serial two-fold dilutions of sera were tested; titres were expressed as the highest dilution giving a cytotoxicity index of at least 50. Cytotoxicity indices were computed by the following formula (Shirai, Yoshiki & Mellors, 1972): CI = [(percentage of dead cells with serum − percentage of dead cells with complement)/(100% − percentage of dead cells with complement)] × 100.

Sucrose density gradient ultracentrifugation

Four pooled serum samples of 0·2 ml volume each were subjected to ultracentrifugation in a 10–35% sucrose density gradient (0·15 M NaCl, pH 8·0) using an SW 40 rotor (Beckman Instruments, Incorporated, Spinco Division, Palo Alto, California). The sera were pooled from three to four mice in each of four different groups: (1) 5½-month-old B/W controls; (2) 5½-month-old B/W GVH; (3) 7½-month-old B/W controls; (4) 7½-month-old B/W GVH. The centrifugation was at 39,000 rev/min for 20 hr. Three proteins of known sedimentation constants were run in a companion gradient and served as reference markers. The 13·5-ml gradients were collected dropwise into fifty fractions and analysed for antibody to DNA. The gradient fractions were also analysed for antibody to rA–rU; however, the radioactivity retained on the filters in these assays was too low to permit interpretation.

RESULTS

Anti-RNA and Anti-DNA assays

The levels of the anti-RNA and anti-DNA antibodies in the recipient B/W mice injected with donor spleen cells are shown in Table 1. Initial mean values for anti-DNA and anti-RNA were similar in GVH (injected with parental NZB cells) and control (injected with syngeneic cells) groups. The mean ct/min of the GVH group was greater than the control group for both antibodies at every post-treatment interval tested. In both antibody assays the mean of all values after initiation of treatment was significantly greater in the GVH group than in the control group.

TABLE 1. Anti-DNA and anti-RNA in NZB/NZW F1 mice after chronic GVH disease

Antibody activity	Transplanted cells	Mean ct/min				
		Pre-treatment values	Months post-treatment 1	2	3	All post-Rx values
DNA	Control	95	130	166	207	168
DNA	GVH	94	159	212*	245	205*
RNA	Control	54	73	87	99	86
RNA	GVH	55	117	119	118	118*

* Statistically significant ($P<0.05$).

The age-related increase in mean values for anti-DNA in both control and GVH groups is shown in Fig. 1. The anti-DNA response was accelerated in the GVH compared to the control group. This difference became significant after two months of parental spleen cell injections.

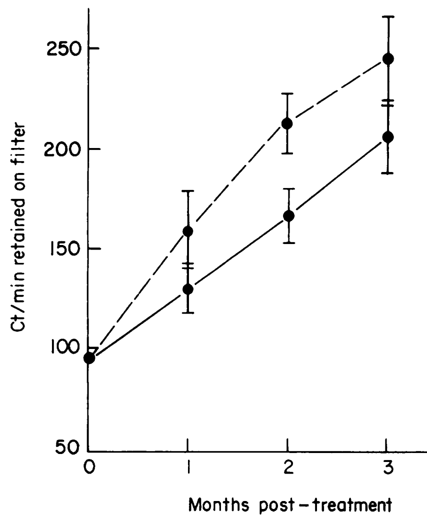

FIG. 1. Acceleration of anti-DNA antibody formation in chronic GVH disease of NZB/NZW mice. The appearance of antibodies to [^{3}H]DNA in the serum of NZB/NZW F1 mice injected with syngeneic (control) (● — ●) or parental (GVH) (● – – ●) lymphocytes.

Thymocytotoxic antibody

Thymocytotoxic antibody titres were similar in both GVH and control groups prior to the injection of spleen cells. However, titration of sera obtained 3 months after initiation of spleen cell injections revealed significantly greater levels of antibody to thymocytes in GVH mice. As shown in Table 2, nine out of ten mice in the GVH group had titres of 1 : 16 or greater compared to only one out of seven mice in the control group. Thus, thymocyto-

TABLE 2. Thymocytotoxic antibody titres in NZB/NZW F1 mice after chronic GVH disease

	Reciprocal of serum dilution			
Group	8 or less	16	32	64
Control	6	1	0	0
GVH	1	2	2	5

toxic antibodies as well as anti-nucleic acid antibodies were accelerated by the induction of GVH.

Sucrose density gradient ultracentrifugation

Anti-nucleic acid antibodies in B/W mice can be separated into 7S and 19S fractions by sucrose density gradient ultracentrifugation. At 5½ months of age, the GVH sera showed a marked increase in 7S anti-DNA compared to age-matched control sera (Fig. 2). There

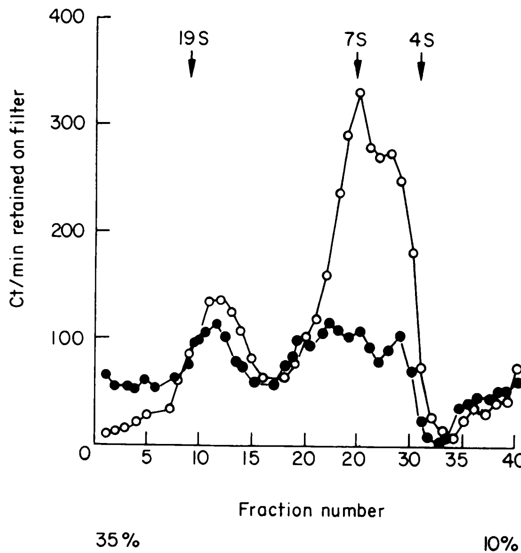

FIG. 2. Accelerated switch from 19S to 7S anti-DNA following GVH disease. Sucrose density gradient ultracentrifugation of pooled 5½-month-old NZB/NZW F1 sera from control (●) or GVH (○) mice. Gradient fractions were collected and assayed for antibodies to [^{3}H]DNA.

was very little difference in the 19S antibody. Five-month-old B/W mice which received no spleen cell injections had an anti-DNA binding pattern similar to that of the control group. Thus, an early effect of the GVH response may be to accelerate the switch from 19S to 7S anti-DNA production. At 7½ months, there is a marked increase in both 19S and 7S anti-DNA in the GVH compared to the control sera (Fig. 3).

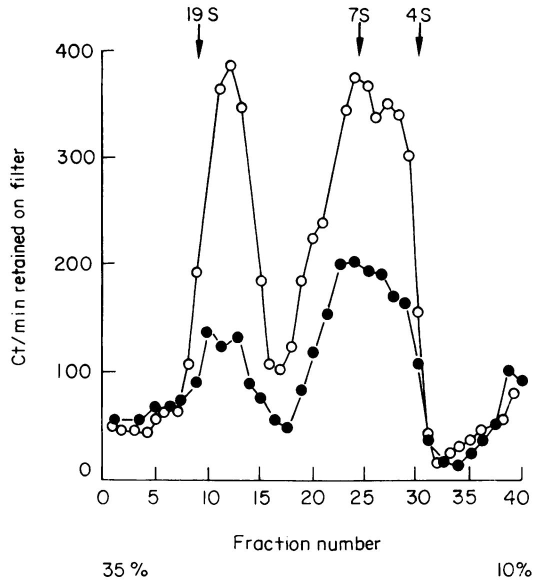

FIG. 3. Increase in anti-DNA following GVH disease. Sucrose density gradient ultracentrifugation of pooled 7½-month-old NZB/NZW F1 sera from control (●) or GVH (○) mice. Gradient fractions were collected and assayed for antibodies to [^{3}H]DNA.

DISCUSSION

The superimposition of GVH disease upon the autoimmune disorder of B/W mice results in accelerated production of antibodies reacting with nucleic acids and with thymocyte cell surface antigens. This acceleration of two different types of autoantibodies suggests a generalized phenomenon acting on a mechanism central to the generation of autoimmunity.

This central mechanism may depend upon a delicate balance between suppressor and helper activity of thymocytes (Allison, Denman & Barnes, 1971). The thymus is an important regulator of the immune activity of B lymphocytes and antibody-producing plasma cells. Thymocytes can co-operate positively (Claman, Chaperon & Triplett, 1966; Katz & Benacerraf, 1972) with B cells for maximum antibody production (helper activity), or can co-operate negatively (Gershon *et al.*, 1972; Droege, 1971; Baker *et al.*, 1970) to dampen antibody responses (suppressor activity). A deficiency of suppressor activity may contribute to the development of autoimmunity in NZB and B/W mice (Barthold, Kysela & Steinberg, 1974; Talal, *et al.*, 1974). Thus, the spontaneous production of auto-antibodies in B/W mice may result from a genetically determined thymocyte imbalance in which helper activity is excessive relative to suppressor function.

The induction of a GVH reaction can substitute for thymic helper function and result in a phenomenon known as the 'allogeneic effect'. For example, genetic non-responder mice can only make an IgM antibody response to certain synthetic polypeptide antigens. Responder mice go on to make an IgG response provided that T cells are present. Neonatal thymectomy of responder mice (Mitchell, Grumet & McDevitt, 1972) converts them to a 'non-responder' pattern (i.e. IgM response only). Conversely, the injection of parental

lymphoid cells into F1 non-responder mice overcomes the genetic barrier and allows them to make an IgG as well as IgM response (Ordal & Grumet, 1972).

In addition, the injection of allogeneic cells has been shown to circumvent the usual requirement for carrier specificity in secondary anti-hapten antibody response to hapten–carrier conjugates. For example, in guinea-pigs the injection of immunocompetent allogeneic lymphoid cells prepares DNP–OVA-primed recipients for a striking secondary anti-DNP response to DNP–BGG (Katz *et al.*, 1971).

A similar 'allogeneic effect' can be demonstrated *in vitro* following interaction of histo-incompatible thymocytes. Culture supernatants obtained from mixed lymphocyte reactions can substitute for helper T cells and restore the antibody response of spleen cell cultures from adult thymectomized, bone marrow repopulated mice (Dutton & Hunter, 1974). Both the frequency of antibody-responding B cells as well as the average clone size are increased by these allogeneic supernatants.

Thus, the GVH reaction results in augmentation of antibody responses and promotes the switch from IgM to IgG. These are exactly the results we found for autoantibodies when GVH disease was induced in B/W mice. We conclude that the allogeneic influence further contributed to the thymic imbalance in B/W mice by favouring the generation of additional helper activity. The mechanism of this helper activity is unknown but may involve specific or non-specific factors released by T cells. A non-dialysable factor of molecular weight 27,000 has been found in allogeneic supernatants (Dutton & Hunter, 1974; Hunter & Kettman, 1974). It is possible that the IgM to IgG switch is promoted by an immunoglobulin (IgT) on the T-cell surface which is released in an antigen complex and then attaches to macrophages (Feldmann, 1972; Katz & Benacerraf, 1972). It is also theoretically possible that suppressor activity was further diminished by the allogeneic interaction, although there is no precedent for this.

Activation of leukaemia virus as a consequence of allogeneic interaction could also contribute to accelerated autoantibody formation by generating T-cell helper activity (Allison, Denman & Barnes, 1971). GVH disease has been demonstrated to activate latent murine leukaemia viruses (MuLV) (Hirsch *et al.*, 1970); indeed, cell-free extracts of tissues obtained from mice undergoing GVH disease are oncogenic and have been shown to contain MuLV (Armstrong, Black & Richards, 1972). Interferon treatment of GVH animals reduces virus production in these animals and modifies the nature of the GVH itself (Hirsch *et al.*, 1973). Since New Zealand mice harbor a C-type virus particle which is easily demonstrable by electron microscopy (Mellors & Huang, 1966), activation of this or other viruses in our B/W mice may be another mechanism whereby autoimmunity is accelerated by the GVH reaction.

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