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Increased survival times of New Zealand hybrid mice immunosuppressed by graft-versus-host reactions

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SUMMARY

New Zealand mice develop autoimmune disease usually accompanied by glomerulonephritis. A graft-versus-host reaction was induced in New Zealand Black × New Zealand White F_1 hybrid mice by administration of New Zealand White spleen cells. The mice so treated had diminished antibody responses to both an exogenous antigen (sheep red blood cells) and an endogenous antigen (native DNA). They had much less glomerulonephritis and increased survival times compared to unmanipulated controls, apparently due to immunosuppression. Similar hybrid mice treated with high doses of cyclophosphamide (70 mg/kg/week) were more immunosuppressed than mice with graft-versus-host reactions and had even greater survival times.

INTRODUCTION

New Zealand black (NZB) mice develop a lupus-like syndrome associated with autoantibodies, including antinuclear antibody (ANA) and Coombs' antibody, and fatal immune complex glomerulonephritis. The [NZB × New Zealand white (NZW)]F_1 hybrid (NZF_1) have particularly severe glomerulonephritis, usually fatal in the female by age 9 months (Lambert & Dixon, 1968). In general, F_1 hybrid mice receiving parental lymphoid cells develop a graft-versus-host reaction (GVHR). GVHR is sometimes accompanied by autoantibody formation including ANA and anti-erythrocyte antibody (Fialkow, Gilchrist & Allison, 1973; Streilein, Stone & Duncan, 1975). The similarity between these two disease models suggests that GVHR in NZF_1 mice might accelerate their autoimmune disease, hastening mortality. Indeed, a recent study by Goldblum *et al.* (1975) indicated that the severity of autoimmunity in NZF_1 mice was increased by GVHR induced by weekly injections of NZB spleen cells, although the effects on renal disease and mortality were not gauged.

On the other hand, it is well known that the GVHR suppresses both humoral (Möller, 1971) and cellular (Lapp & Möller, 1969) immune responses. Since chemical immunosuppression reduces autoimmune reactions and prolongs survival in NZF_1 mice (Gelfand & Steinberg, 1972; Hahn *et al.*, 1973), GVHR induced immunosuppression also might increase their survival. Therefore we induced chronic GVHR in female NZF_1 mice and compared their immunologic status, glomerulonephritis and survival rates to those in untreated controls and in mice chemically immunosuppressed with high doses of cyclophosphamide (CP).

NZ mice harbour murine leukaemia virus(es) (MuLV). Two MuLV proteins, p30, the major internal structural component of MuLV, and GP70, the major glycoprotein constituent of the virion envelope, can be easily quantitated and GP70 apparently contributes to the immune complex deposits found in the NZF_1 kidneys (Yoshiki *et al.*, 1974). We examined all mice for the occurrence of lymphoma; in addition, we measured serum levels of these two viral products.

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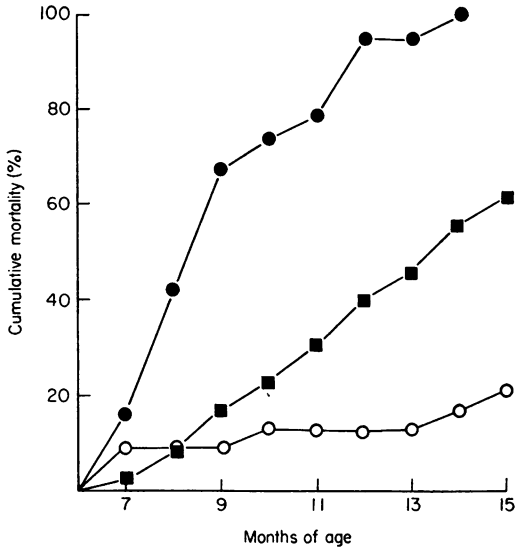

FIG. 1.

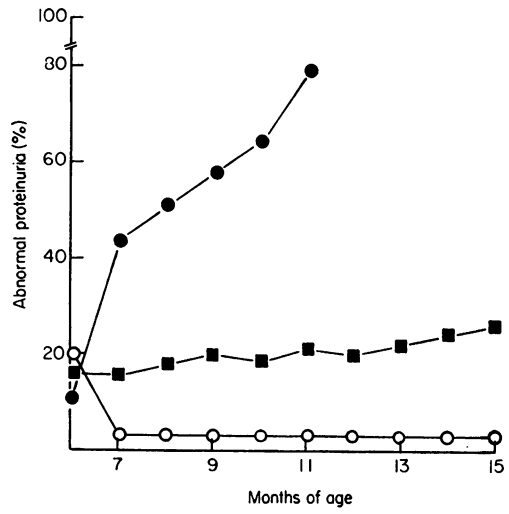

FIG. 2.

FIG. 1. Cumulative mortality of NZF_1 mice. ■, GVHR; ○, CP; ●, control. Mortality in GVHR-induced mice was markedly reduced compared to control mice, although it was not as low as in the CP-treated group.

FIG. 2. Abnormal proteinuria in NZF_1 mice. ■, GVHR; ○, CP; ●, control. Each value includes proteinuric mice which had died, plus any mice still having proteinuria at the month observed. All proteinuric control mice died by 11 months.

MATERIALS AND METHODS

Mice. NZW and NZF_1 mice were bred and maintained in our own colonies. Experimental and control mice were housed in the same area. Only female mice were used in the experiment.

Induction of GVHR and chemical immunosuppression. Spleen cell suspensions from 5-month-old NZW mice were prepared by gently mincing spleens on stainless steel mesh, and then washing them in balanced salt solution. GVHR were induced in each of fifty-two 5–6-month-old NZF_1 mice by single intravenous injections of 3×10^7 viable (by trypan blue exclusion) spleen cells. Twenty-four NZF_1 mice received CP (Mead Johnson Laboratories, Evansville, Indiana), 70 mg/kg of body weight/week, intraperitoneally. Nineteen unmanipulated NZF_1 mice served as controls.

Antibody assays. Immune responses were evaluated by assaying for antibodies to both an exogenous antigen, anti-sheep red blood cell (SRBC) antibody, and endogenous antigens, Coombs' antibody, ANA, and anti-native DNA (nDNA). Mice were bled by orbital sinus puncture at initiation of the experiment and monthly thereafter. Two months after the start of the experiment all mice were injected intraperitoneally with 5×10^8 SRBC (Colorado Serum Co., Denver, Colorado) and tested for Coombs' reactivity and SRBC haemagglutination response 12 days later. Haemagglutination responses were measured in microtitre plates (1:10 – 1:640). Titres of ANA (1:3 – 1:192) were determined monthly by indirect immunofluorescence, using mouse kidney sections and fluorescein-conjugated rabbit anti-mouse gamma globulin.

Antibody to nDNA was determined in all mice at approximately 8 months of age using a modification of the Farr technique. Calf thymus DNA (Worthington Biochemical Corp., Freehold, New Jersey) was labelled with ^{125}I (Commerford, 1971). nDNA was isolated by hydroxyapatite chromatography (Bernardi, 1971). To 50 μl ^{125}I-nDNA (1 μg/ml) was added to 50 μl of serum, diluted 1:10. Tubes, in triplicate, were incubated 16 hr at 4°C. After incubation, an equal volume of 50% SAS was added. The specimens were then thoroughly mixed and incubated 30 min at 4°C. Tubes were centrifuged at 12,000 *g* for 30 min, the supernatant aspirated and tubes counted. The ^{125}I-nDNA solution contained ^{131}I-gamma globulin (10 μg/ml) as a precipitation control and ^{22}Na as an entrapment control. Nonspecific precipitation, determined by using 10% decomplemented normal rabbit sera, was 3–4%; this percentage was subtracted from the total binding percentage to give the actual percentage for each sample.

MuLV antigen levels. Serum p30 and GP69/71 levels were determined by radioimmunoassay before the experimental procedures were initiated and 6 months thereafter (Oroszlan *et al.*, 1972; Parks & Scolnick, 1972).

Assay for proteinuria. Urine was tested monthly for 24 hr protein excretion by measuring turbidity induced by addition of 3% sulfosalicylic acid. Mice were considered to be proteinuric when they excreted ≥ 2 mg/24 hr, since forty 3-month-old female NZF_1 mice excreted mean $0{\cdot}97\pm0{\cdot}96$ (s.d.) mg/24 hr.

Immuno- and histopathologic findings. Mice were autopsied shortly after death, and tissues were fixed in Bouin's solution. Nine months after beginning the experiment (when all control mice were dead), all surviving mice with induced GVHR and mice given CP were killed. Renal pathologic abnormalities were graded on a scale of 0 – 3, as follows: 0, normal; 1,

occasional abnormal glomeruli with increased mesangial matrix, focal hypercellularity and/or mild basement membrane thickening, with normal tubules; 2, diffuse glomerular involvement, with mesangial hypertrophy, hypercellularity and basement membrane thickening, with tubular damage; 3, most or all glomeruli abnormal, frequently with fibrinoid and sclerotic changes, with marked tubular injury. Portions of kidneys were snap-frozen, treated with fluorescein-conjugated rabbit anti-mouse Ig, and examined by immunofluorescence microscopy (Wilson & Dixon, 1974). The amount and intensity of fluorescent glomerular Ig deposits were graded on a scale of 0 to 4+.

Statistical analyses. Statistical comparisons were performed by the Student *t*-test and Chi square analyses.

RESULTS

Survival

Both GVHR and CP treatment decreased the mortality of the NZF$_1$ mice noticeably within 2 months ($P<0{\cdot}05$ for GVHR) of beginning treatment (Fig. 1). Thereafter, both treatments significantly reduced mortality ($P<0{\cdot}01$). CP was more effective and beginning at the twelfth month caused a significant reduction ($P<0{\cdot}05$) in mortality over that produced by GVHR. By 12 months only 1/19 control mice survived while 20/52 and 19/24 GVHR and CP treated mice respectively survived until killed at 15 months.

Renal disease

Proteinuria. The incidence of increased proteinuria was reduced in the GVHR and CP-treated mice paralleling the reduction in mortality (Fig. 2). Whereas 79% (15/19) of the control mice became proteinuric (all before 11 months of age), only 26% (13/52) of the mice with GVHR and 4% (1/24) of the CP-treated mice had developed increased proteinuria by 15 months of age. Statistical analysis at 11 months, when meaningful comparison was possible, yielded the following: GVHR *v.* control group, $P<0{\cdot}01$; CP *v.* control group, $P<0{\cdot}01$; CP *v.* GVHR group, $P<0{\cdot}05$. Of interest, CP appeared to decrease the incidence of proteinuria observed in the baseline determinations (Fig. 2).

Renal histology. All mice that died during the course of the experiment exhibited renal damage; the mean$\pm$s.d. histologic score for these mice was $2{\cdot}12\pm0{\cdot}96$. In contrast, surviving mice had much lower scores; $0{\cdot}78\pm0{\cdot}73$ in the GVHR group and $0{\cdot}68\pm0{\cdot}58$ in the CP-treated animals. Both scores were significantly lower than that of the dead mice ($P<0{\cdot}01$). (They were not significantly different from each other.)

Immunopathology. Granular immune complex deposits of Ig were seen in glomeruli of all kidneys. These deposits were prominent in all mice dying during the study (score $3{\cdot}57\pm0{\cdot}67$) and equally so in

TABLE 1. Reciprocal GMT of ANA in NZF$_1$ female mice

Months of age	Control mice	GVHR mice	CP-treated mice
6	11·7 (19)	12·6 (52)	11·0 (24)
7	50·3 (16)	13·5 (51)	5·5 (22)
8	37·1 (11)	29·9 (48)	14·3 (22)
9	9·0 (6)	25·1 (43)	2·1 (22)
10	21·1 (5)	8·3 (40)	1·3 (21)
11	13·0 (4)	25·6 (36)	1·0 (21)
12	—	22·6 (31)	0·4 (21)
13	—	49·6 (28)	1·5 (21)
14	—	46·4 (23)	1·0 (20)
15	—	60·8 (20)	4·8 (19)
Mean$\pm$s.d. through 11 months	23·7$\pm$16·5	19·17$\pm$8·78	5·87$\pm$5·59

Numbers of mice studied are given in parentheses.

surviving mice with GVHR (3·41±0·69). The deposits were significantly reduced in surviving CP-treated mice compared to the other two groups, 1·68±0·89, $P<0·01$.

Immune responses

Response to SRBC. The reciprocal geometric titre (GMT) of the haemagglutination response to immunization with SRBC was 126·3±3·7 in the control group. The GVHR group had a somewhat lower response, the reciprocal GMT being 72·4±9·5. The CP group had an even lower reciprocal GMT: 26·9±6·3. The Student *t*-test yielded the following results: GVHR *v.* control group, $P<0·02$; CP *v.* control group, $P<0·001$; CP *v.* GVHR group, $P<0·05$.

Coombs' antibody response. The incidence of Coombs' antibody at approximately 8 months of age was similar in controls and mice with GVHR; 45% (5/11) in the control group and 44% (18/41) in the GVHR group. In contrast, none (0/22) of the CP-treated mice had Coombs' antibody; this incidence was significantly different from that in the other two groups: $P<0·01$.

ANA. The monthly reciprocal GMT for ANA of all three groups are given in Table 1. Because of the high mortality of the control group, statistical testing was done for 11 months. The mean in the GVHR group, 19·2±8·9, was not significantly different from that in the control group, 23·7±16·5. However, the mean for the CP-treated group was significantly lower than both of these: 5·9±5·6, $P<0·05$.

Antibody to nDNA. Mean % binding of nDNA by the control group was 9·97±6·16. Binding was reduced in the GVHR group, 5·15±5·42, and it was even further reduced in the CP-treated group, 1·99±2·15. Student *t*-testing gave these results: GVHR *v.* control group, $P<0·05$; CP *v.* control group, $P<0·01$; CP *v.* GVHR group, $P<0·05$.

TABLE 2. Serum MuLV antigen levels

	6 months of age		12 months of age	
	P30 (ng/ml)	GP69/71 (μg/ml)	P30 (ng/ml)	GP69/71 (μg/ml)
Control	79±60	32±18	62±40*	42±23*
GVHR	53±27	26±10	55±35	43±35
Cytoxan	76±65	28±9	80±30	27±5

* These values are at 9 months of age, since almost all control mice were dead at 12 months.

Lymphoma/MuLV titres

Lymphoma. Lymphoblastic lymphomas were noted in one control mouse, three mice with GVHR but no CP-treated mice. The affected control mouse survived longest of its group, 14 months, and was the only control mouse to survive past 12 months. It had titres of autoantibody comparable to those of the CP-treated group and significantly lower than those of the control group: mean reciprocal GMT of ANA, 3·38 ($P<0·01$); % nDNA binding, 0·57, $P<0·01$. Although it had 3+ renal deposits of Ig its renal histologic index was only 1. The three lymphomatous mice with GVHR all survived to the end of the study; their immunologic parameters were the same as those of the GVHR group at large.

MuLV levels. Mean MuLV levels for p30 and GP69/71 are given in Table 2. There is no significant difference between mean levels of either p30 or GP69/71 regardless of treatment. Levels in mice with lymphomas were not significantly different from mean values.

DISCUSSION

Rather than augmenting autoimmune disease in NZF_1 mice, the GVHR decreased it, apparently by

immunosuppression. NZF_1 mice undergoing a chronic GVHR enjoyed significantly increased survival times compared to control mice, as a result of a reduction in the severity of autoimmune immune complex induced glomerulonephritis. In turn, the amelioration of glomerulonephritis was apparently due to immunosuppression. Compared to control mice, the mice with GVHR had lower antibody responses to an exogenous antigen, SRBC, and an autoantigen, nDNA. GVHR failed to suppress ANA or Coombs' antibody. Several groups of investigators have previously shown that these autoantibody responses are difficult to suppress with drugs, even when diminution of responses to exogenous antigens and nDNA can be demonstrated (Hahn *et al.*, 1973; Walker & Bole, 1975). GVHR did not diminish the intensity of glomerular Ig deposits observed at autopsy. It should be recalled, however, that the study was not designed to evaluate the rate of accumulation of glomerular immune complex deposits. The mice were examined only when they had succumbed of glomerulonephritic complications or in the surviving GVHR mice after having been exposed to potential nephritogenic immune complexes for at least 4 months longer than their unmanipulated counterparts.

Mortality, renal disease and antibody responses in mice with GVHR were intermediate between the control group and the CP-treated group. The GVHR supplied a modest degree of immunosuppression roughly equivalent to that provided by CP, 4·5 mg/kg/day (Gelfand & Steinberg, 1972), or prednisolone, 5 mg/kg/day (Hahn *et al.*, 1973). High doses of CP (70 mg/kg/week) are obviously very effective in treating the autoimmune disease of NZB/W mice. Immune responses, including Coombs' antibody and ANA, that had been unaffected by the GVHR, were significantly depressed by CP and renal Ig deposition was also decreased. Using the same high dose of CP, Hoffsten & Dixon (1973) showed a reduction in renal disease and glomerular immune complex Ig deposition in mice chronically infected with lymphocytic choriomeningitis virus.

The incidence of lymphoma in all mice was very low. Of interest, the only control mouse with a lymphoma had a very low autoantibody titre, relatively mild glomerulonephritis, and survived longer than any other control mouse; it may have been immunosuppressed by its tumour burden. Though the GVHR may increase the incidence of lymphomas in some murine strains, the NZF_1 mice did not respond in this fashion. CP treatment did not increase the incidence of lymphomas either. Lymphomas reportedly developed in CP-treated mice previously, but not until late in life: 16–17 months of age in one study (Walker & Bole, 1973) and 19 months in another (Hahn *et al.*, 1975). Our mice were killed at 15 months of age; they might simply not have lived long enough to develop lymphomas.

Levels of circulating MuLV antigens were unaffected by immunosuppression with either the GVHR or CP. Immunosuppression also failed to alter circulating virus titres in mice with chronic lymphocytic choriomeningitis infections (Hoffsten & Dixon, 1973). Apparently, nonimmunologic mechanisms are of primary importance in controlling such levels.

The palliative effect of the NZW spleen cell induced GVHR on autoimmune nephritis which we found is at variance with the increased autoantibody formation of NZB induced GVHR in NZF_1 mice described by Goldblum *et al* (1975). While the difference in results might be related to the two kinds of parental cells used, preliminary experiments in our laboratory have also shown slightly increased survival of NZF_1 mice given NZB parental spleen cells. While the latter mice have a higher incidence of early, fatal GVHR than those given NZW spleen cells, the survivors have slightly prolonged longevity over their unmanipulated counterparts. The difference in results may also have originated from the protocol followed by Goldblum *et al.* (1975), which employed weekly injections of NZB cells to induce the GVHR. It may be that the disparity in the effect of the GVHR induced by cells of each parental strain in the two studies is more apparent than real and merely reflects the dissimilar parameters measured in the two experiments.

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