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IMMUNOBIOLOGY OF THE AUTOANTIBODY RESPONSE

I. CIRCULATING ANALOGUES OF ERYTHROCYTE AUTOANTIGENS AND HETEROGENEITY OF THE AUTOIMMUNE RESPONSE OF NZB MICE

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SUMMARY

Anti-erythrocyte autoantibodies of two distinct antigenic specificities can be recovered from immunoglobulin-coated NZB erythrocytes. These autoantibodies react with either exposed (X) or hidden (HB) antigenic determinants on murine red cells and both types can be neutralized by mouse plasma. Antibody neutralization by plasma is dependent on two distinct plasma activities which appear to be soluble analogues of the erythrocyte surface autoantigens X and HB. The soluble erythrocyte antigens SEA-X and SEA-HB can be separated by molecular exclusion chromatography, with approximate sizes of 2×10^5 for SEA–X and $\geqslant 5 \times 10^6$ for SEA-HB. The presence of these soluble erythrocyte autoantigens in the plasmas of 'autoimmune' NZB mice has to be considered in relation to the immunologic homeostasis of these autoantibody responses and pathogenesis of both the autoimmune haemolytic anaemia and the autologous immune complex disease of these mice.

Individual anti-erythrocyte autoantibodies of both anti-X and anti-HB types exhibit discrete and individually distinctive differences in affinity for autoantigen and also in respect to antigenic determinant specificity. These results suggest that factors which control the genetic selection of these responses in an inbred strain do not prescribe a single specific B lymphoid cell clone; but rather it appears that a number of different autoimmunocompetent B lymphocyte clones with differing affinity and determinant specificity for given autoantigen molecules may be derepressed by other events in the recruitment of the autoantibody responses.

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INTRODUCTION

New Zealand black (NZB) mice, utilized widely in exploration of the immunobiology of autoimmunity (Howie & Helyer, 1968; Long, Holmes & Burnet, 1963; Staples & Talal, 1969; Mellors, Aoki & Huebner, 1969; Rodney, Good & Yunis, 1971; Wilson, Warner & Holmes, 1971), spontaneously develop autoantibody responses to autologous tissue antigens including DNA (Norins & Holmes, 1964) and erythrocyte surface antigens (Bielschowsky, Helyer & Howie, 1959; Holborow, Barnes & Tuffrey, 1965; Costea, Yakulis & Heller, 1970; Linder & Edgington, 1972a). Warner & Wistar (1968) have demonstrated that the autoantibody response to the erythrocyte is polyclonal in respect to heavy chain class. We have shown that erythrocyte autoantigen is present not only on the erythrocyte surface but also in murine plasma as soluble erythrocyte autoantigen (SEA) (Linder & Edgington, 1971a). The existence of two discrete and independent pathogenetic erythrocyte surface autoantigens and their homologous autoantibodies has been demonstrated more recently (Linder & Edgington, 1972 a and b). We now report the existence of two SEAs, in the plasma of mice; each appears to represent a soluble analogue of one of the previously described erythrocyte surface bound autoantigens. It is further suggested that these represent two separate molecules, and thus must be considered independently in evaluation of their potential role in the immunobiology of anti-erythrocyte autoantibody responses, in the mediation of cell injury, and in the genesis of autologous immune complex disease. The autoantibody responses to these two erythrocyte autoantigens appear heterogeneous in respect to affinity and antigenic determinant specificity, which in turn suggests the recruitment of a heterogeneous population of autoimmunocompetent B lymphocytes in NZB mice.

MATERIALS AND METHODS

Animals

NZB mice were originally obtained from the Laboratory Animal Centre, M.R.C., Surrey, and have been maintained by brother–sister mating. C57Bl/6 mice were acquired from Jackson Laboratories, Bar Harbor, Maine, and BALB/c mice from Strong Laboratories, San Diego, California. (NZB × BALB/c F_1 hybrid mice were bred in our laboratory.

Red cell and plasma samples

Mice were bled from the retro-orbital plexus into heparinized capillary tubes. Erythrocytes were separated from the plasma by centrifugation for 5 min at 1000 *g* and were washed three times with greater than 500 volumes of 0·14 M NaCl, 0·01 M sodium phosphate, pH 7·2 (PBS). Plasma samples for absorption experiments and molecular exclusion chromatography were first passed through 0·45 μm Millipore filters.

Anti-erythrocyte autoantibodies

A mixture of anti-HB and anti-X anti-erythrocyte autoantibodies (Linder & Edgington, 1972b), bound *in vivo* to erythrocytes of NZB mice, were isolated from four times washed direct Coombs' positive red cells by a modification of the method of Weiner (1957). After lysis by freezing and thawing, precipitation was performed at −20°C with 50% ethanol. The precipitate was centrifuged at 1000 *g*, −8°C for 20 min, washed with distilled water at 0°C and again centrifuged. The autoantibody was eluted from the precipitate with PBS at 37°C and recovered in the supernatant following centrifugation for 20 min at 1000 *g*.

Anti-HB anti-erythrocyte autoantibodies free from anti-X activity (Linder & Edgington, 1972b) were prepared in two ways. *In vivo bound* autoantibodies which were eluted from autoantibody coated NZB red cells contained both anti-X and anti-HB autoantibodies. These preparations were absorbed once for 15 min at 37°C with an equal volume of three times washed intact C57Bl red cells. The absorption was sufficient to remove all detectable autoantibodies of anti-X specificity and the absorbed preparation exhibited only specific anti-HB activity. Anti-HB autoantibodies were also prepared by absorption and subsequent elution of *in vivo free* autoantibodies on bromelin (enzyme)-treated erythrocytes; plasmas with anti-HB autoantibody activity were pooled and three parts of plasma was absorbed with one part packed enzyme-treated erythrocytes for 30 min at 37°C. Anti-HB autoantibodies were eluted from the antibody-coated erythrocytes by the modified method of Weiner (1957) as described above.

Assay of anti-erythrocyte autoantibodies

Anti-erythrocyte autoantibodies were assayed by indirect Coombs' haemagglutination and indirect enzyme augmented haemagglutination techniques as previously described (Linder & Edgington 1972a). All assays utilized the basic ultramicro-haemagglutination technique (Linder & Edgington 1971b), employing 10^4 intact or enzyme-treated (Bromelase, Dade Reagents, Inc., Miami, Fla.) C57Bl/6 mouse erythrocyte in 1 μl PBS as target cells. All antibody and antigen titres were expressed on a modified $\log_2$ basis as the reciprocal of the highest doubling dilution at titration end-point. Negative was expressed as 0, positive at neat concentration as 1, and positive at a 1:2 dilution as 2, etc.

Haemagglutination inhibition assays for HB and X autoantigens

Cells, plasma and plasma fractions were assayed for HB and X autoantigens by inhibition of specific haemagglutination by NZB anti-HB or anti-X autoantibodies. Minor modifications of the originally described haemagglutination inhibition assays (Linder & Edgington, 1972a) were employed to meet specific and varying needs for sensitivity and quantitation, and to accommodate experimental design. Titres were expressed on a modified $\log_2$ basis from the reciprocal of the highest dilution end-point after correction of volume variation between assay modifications.

Assays for X autoantigens

Anti-erythrocyte autoantibodies reacting with the exposed (X) murine erythrocyte autoantigen were demonstrated specifically by the indirect Coombs' method as previously described (Linder & Edgington, 1972a). Inhibition of this anti-X reaction permits assay of X antigen activity. Assay modification A: 5 μl of sample was first incubated with 5μl of anti-X autoantibody at 37°C for 15 min, the mixture was serially diluted, and haemagglutination was determined by the indirect Coombs' assay. Cross titrations were performed with 5 μl serial dilutions of the test antigen, followed by incubation with 5 μl of anti-X autoantibody and subsequent assay of anti-X activity was utilized for more quantitative assessment of the cell bound and soluble X autoantigenic expression. The results of these latter assays are presented graphically.

A screening assay (modification B) for plasmas and plasma fractions determined the highest dilution of sample giving a complete inhibition of anti-X autoantibody at an effective titre of 2. Five microlitre of the sample was serially diluted in PBS and 1 μl of the autoantibody with a titre of 1:12 was added to each dilution. Haemagglutination was then

determined by the indirect Coombs' method and the highest dilution of the sample producing complete haemagglutination inhibition was read.

Assays for HB autoantigens

Enzyme-augmented haemagglutination of a mixture of anti-X and anti-HB anti-erythrocyte autoantibodies detects mainly anti-HB autoantibodies (Linder & Edgington, 1972a); complete haemagglutination inhibition of such autoantibody preparations indicates the presence of HB antigen in the sample tested.

A specific assay for HB antigens was also developed using immunochemically purified anti-HB autoantibodies. Anti-HB autoantibodies purified as described above from the *in vivo bound* and the *in vivo free* populations both exhibited the same characteristics in agglutinating bromelin enzyme-treated but not intact or trypsin-treated mouse erythrocytes. The technical aspects of the various haemagglutination inhibition assay modifications for HB autoantigen were as described in assays for X autoantigens (modifications A and B) except that final assay was by direct haemagglutination of enzyme-treated erythrocytes.

Molecular exclusion chromatography

Mouse plasma was fractionated on beaded agarose Biogel A-5 (Bio-Rad Laboratories) in 0·1 M Tris-HCl pH 8·3. Samples of 2–3 ml were fractionated at 4°C on a 2·5 × 90-cm column with a V_0 of 145 ml.

RESULTS

Neutralization of anti-erythrocyte autoantibodies by plasma

Eleven preparations of *in vivo bound* anti-erythrocyte autoantibodies, eluted from Coombs' positive erythrocytes of NZB mice and containing anti-X autoantibody, were used for detection of putative plasma soluble erythrocytes autoantigen X (SEA-X). The indirect Coombs' titres—reflecting the concentration of anti-X autoantibody—varied from 1 to 5 (modified $\log_2$). All eleven anti-X autoantibodies were completely neutralized by pooled plasma from 1–2-month-old NZB mice. The maximum dilution of mouse plasma necessary for complete neutralization of individual anti-X autoantibodies tended to be inversely proportional to the titre of the autoantibody (Fig. 1). Autoantibodies with titre between 3 and 5 were neutralized by plasma at modified $\log_2$ dilutional titre of 6 to 2 respectively whereas three anti-X antibody preparations with titre of 2 and 1 were neutralized by plasma at dilutional titre of 7 and 8 respectively. The differences in concentration of plasma required for neutralization of different anti-X autoantibodies of similar titre suggests differences in the character or binding affinity of the autoantibody responses to the X autoantigen in different NZB mice. The generic capacity of plasma to neutralize anti-X autoantibody suggests the presence of SEA-X.

Sixteen *in vivo* bound autoantibody preparations, containing anti-HB autoantibodies with enzyme-augmented haemagglutination titres of 2 to 6, also were completely neutralized by pooled plasma from young NZB mice. A proportionate though varying ratio between the enzyme-augmented haemagglutinations titre—an indication of anti-HB autoantibody titre —and the maximum dilution of mouse plasma necessary for neutralization of autoantibody activity was observed (Fig. 2). Seven anti-HB autoantibody preparations with titres of 3, were neutralized by plasma at dilutions ranging from 2 to 8. Though a precise concentration of plasma was required for neutralization of each individual anti-HB autoantibody, wide

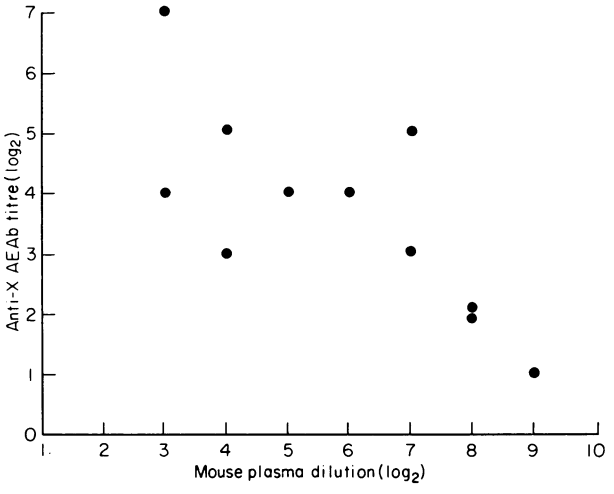

FIG. 1. Neutralization of NZB mouse anti-X erythrocyte autoantibodies by plasma of young NZB mice. The indirect Coombs' titres of eleven *in vivo* bound anti-X autoantibodies (AEAb) are plotted against the maximum dilution of pooled mouse plasma observed to completely neutralize autoantibody activity (anti-X assay modification A).

variations were observed between different NZB mice in respect to this. The varying concentrations of mouse plasma for neutralization of autoantibodies exhibiting similar haemagglutination titres suggests differences in the binding affinity of individual autoantibodies from different NZB mice for soluble erythrocyte autoantigen HB (SEA-HB).

Comparison of erythrocytes surface X and SEA-X autoantigens

The capacity of both erythrocytes and plasma to neutralize anti-X anti-erythrocyte autoantibodies was compared by quantitative haemagglutination inhibition assays using a number of different anti-X autoantibodies.

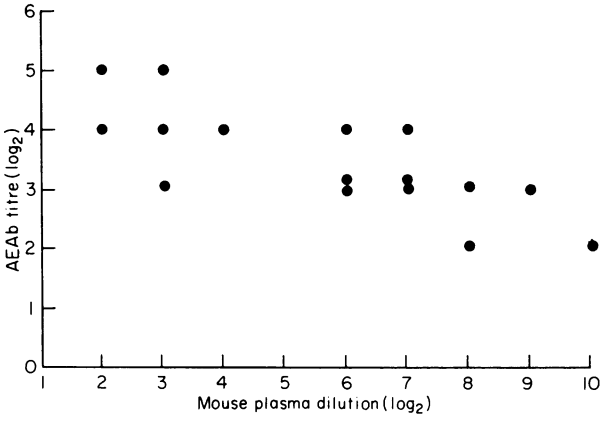

FIG. 2. Neutralization of anti-HB autoantibodies by mouse plasma. Enzyme-augmented haemagglutination is employed to assay anti-HB activity of sixteen autoantibodies (AEAb) eluted from Coombs' positive NZB erythrocytes. AEAb titres are plotted against the maximum dilution of plasma observed to neutralize anti-HB autoantibody activity (anti-HB assay modification A).

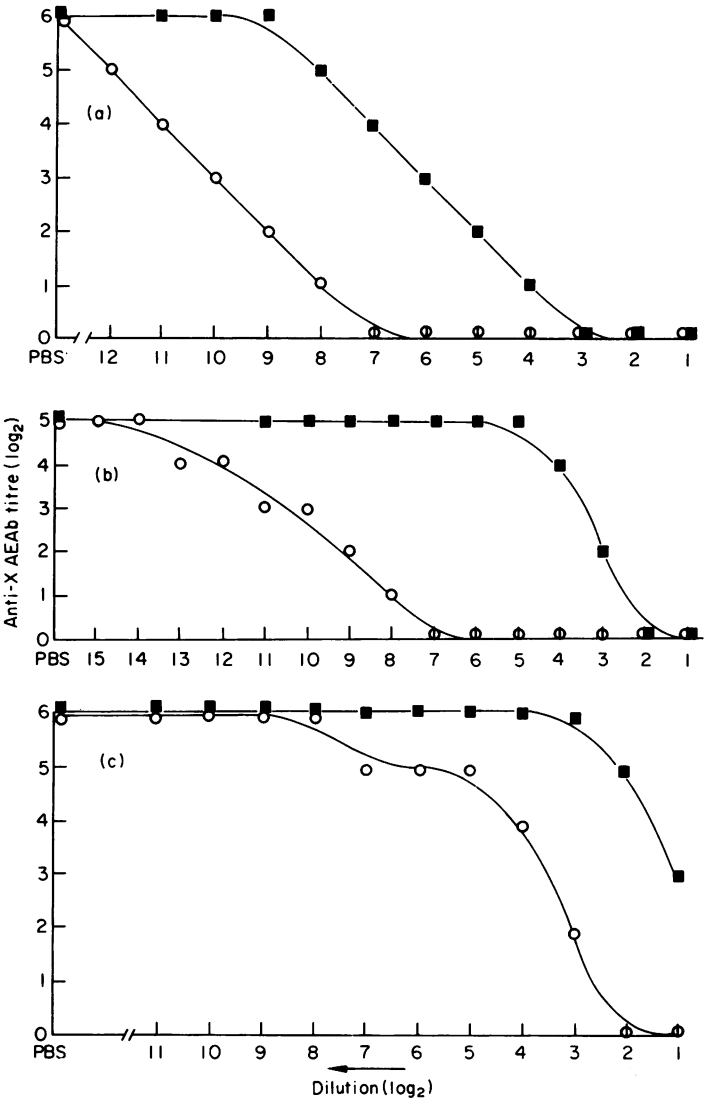

FIG. 3. Comparative inhibition of different anti-X autoantibodies by erythrocyte surface X and plasma SEA-X. (a) Autoantibody 2871. Inhibition of anti-X by erythrocytes (○) or plasma (■) yields similar slopes indicative of similar binding affinity. Fifty per cent inhibition suggests that the plasma pool contained approximately one-sixteenth the concentration of SEA-X by volume that is present on the erythrocyte surface. (b) Autoantibody 3271. Quantitative inhibition of anti-X autoantibody by erythrocytes (○) yielded a non-linear slope suggestive of heterogeneity. Inhibition by plasma (■) gave a quite different inhibition profile indicative of differences in binding of anti-X autoantibodies by SEA-X as contrasted to erythrocyte X. (c) Autoantibody 3371. A minor subpopulation of anti-X autoantibodies are readily neutralized by erythrocytes (○) at low concentration, whereas neutralization of the remaining autoantibodies yields a second slope comparable to that observed with inhibition by plasma SEA-X (■) (anti-X assay modification A).

Three anti-X autoantibodies, with titres of 5 to 7, were quantitatively neutralized in a concentration-dependent fashion by serial dilutions of either plasma or erythrocytes from the same pool of mouse blood (Fig. 3). No significant inter-strain differences were observed between red cells and plasmas from C57Bl/6 and C3H mice. The differing absorption profiles observed with autoantibodies from different individual NZB mice suggest significant differences in binding affinity or determinant specificity between individual anti-X autoantibodies. With some autoantibodies (Fig. 3a) parallel slopes of inhibition by erythrocytes and plasma indicate that such anti-X autoantibodies have a relatively equal affinity for erythrocyte surface X and SEA-X autoantigens. This suggests that some anti-X anti-erythrocyte autoantibodies may react with similar if not identical antigenic determinants of SEA-X and erythrocyte surface X molecules. In all experiments, absorption with erythrocytes was quantitatively more efficient by volume than absorption with plasma. The difference in quantity between erythrocyte surface X and SEA-X estimated when the slopes of the absorption curves are parallel (Fig. 3a) suggests that the concentration of SEA-X is 8–20% of that of the erythrocyte membrane X on a volume basis.

More frequently, the inhibition slope observed upon absorption with erythrocytes was significantly different from that observed for plasma (Fig. 3b). The character of the erythrocyte inhibition slope (more closely approaching 45°) suggests that anti-X autoantibodies may exhibit a higher binding affinity for erythrocyte bound X autoantigen than for SEA-X. Occasionally complex slopes, indicating the presence of more than one autoantibody population, also have been observed (Fig. 3c).

Comparison of erythrocyte surface HB and SEA-HB autoantigens

Differences between erythrocyte bound HB and SEA-HB were observed in respect to neutralization of anti-HB autoantibodies. Purified anti-HB autoantibodies were neither bound to nor neutralized by intact red cells (Fig. 4a). The concentration by volume of erythrocyte surface HB was considerably higher than that of SEA-HB. Occasional anti-HB autoantibody pools, such as illustrated in Fig. 4a, appear to be of reasonably high affinity; however, the more acute inhibition profiles suggestive of low binding affinity, as exemplified in Fig. 4b, were more commonly observed. The majority of anti-HB autoantibodies exhibited slight differences in character of inhibition by erythrocyte HB as contrasted with plasma SEA-HB (Fig. 4). This observation suggests subtle differences in expression or exposure of HB autoantigenic determinants on erythrocytes as compared with SEA-HB. Major differences between inhibition profiles of different anti-HB autoantibodies (Fig. 4) indicate significant differences in these autoantibodies and responsive autoimmunocompetent cells in different mice of the NZB strain.

Separation of Plasma SEA-X and SEA-HB activities by molecular exclusion chromatography

Constituent molecules of NZB mouse plasma were fractionated in respect to size by molecular exclusion chromatography on Biogel A-5 agarose. The elution pattern for plasma pooled from ten NZB mice is shown in Fig. 5. Each effluent fraction was tested for SEA-X and SEA-HB immediately following elution. Included fractions 45–54 representative of molecules of 150–200,000 Daltons contained SEA-X, whereas the excluded protein in fractions 32–36 indicative of a molecular weight of $>5 \times 10^6$ Daltons, contained SEA-HB activity.

The isolated SEA-X and SEA-HB were further characterized by quantitative haemagg-

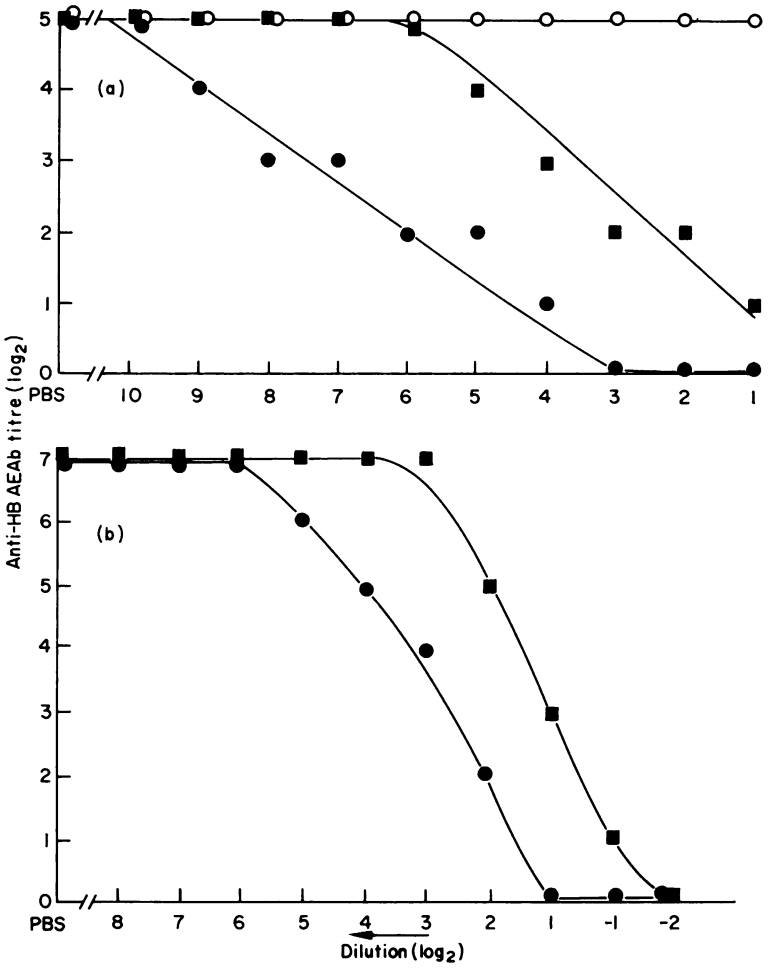

FIG. 4. Comparative inhibition of anti-HB anti-erythrocyte autoantibodies by erythrocyte surface HB and plasma SEA-HB. (a) Absorption of purified pooled anti-HB B4571 with intact erythrocytes (○) produced no inhibition. Absorption with enzyme-treated erythrocytes (●) and plasma (■) demonstrates the marked difference in concentration of HB autoantigen in these two sources. Although generically similar, slight differences in the slope of inhibition are noted. (b) Purified pooled anti-HB B4572. Complete neutralization is effected by both enzyme-treated erythrocytes (●) and plasma (■); however, the slopes of the inhibition profiles appear to differ (anti-HB assay modification A).

lutination inhibition. In one such experiment, 3 ml of pooled plasma from NZB × BALB/c hybrids were chromatographed on Biogel A-5, and the effluent combined to give four fractions levelled A, B, C, and D (Fig. 6a). Each pooled fraction was concentrated to the original 3 ml and assayed for SEA-X by inhibition of anti-X autoantibody (Fig. 6b). Only whole plasma and fraction C neutralized anti-X autoantibodies. Although plasma was somewhat more effective than fraction C, complete inhibition was achieved with both. The inhibition profiles were parallel suggesting immunochemical identity between isolated

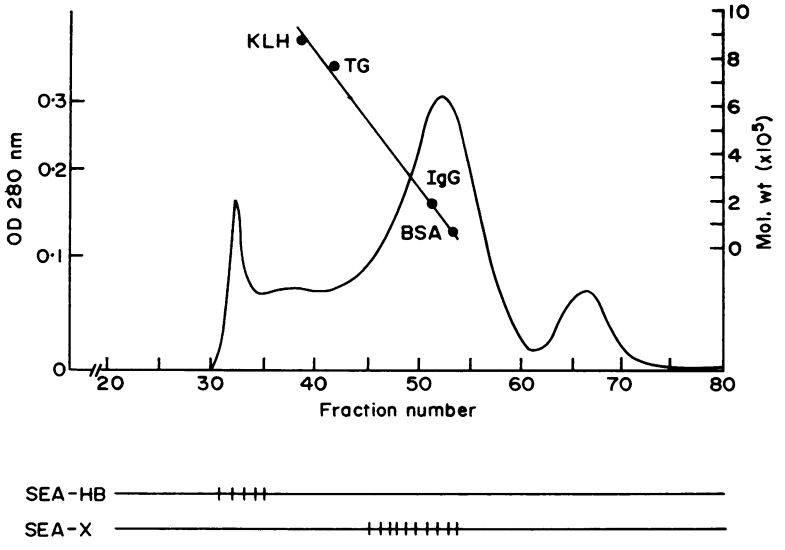

FIG. 5. Fractionation of soluble erythrocyte autoantigen (SEA-X and SEA-HB) activities in plasma by molecular exclusion chromatography on Biogel A-5. SEA-HB activity from 3 ml of plasma was demonstrated in the excluded fractions 32–35, whereas SEA-X activity was observed only in the included fractions 45–54. Each autoantigen determined by assay modification B. Calibration of molecular exclusion behaviour in respect to molecular weight is indicated using as standards Keyhole Limpet haemocyanin (KLH) $8{\cdot}80 \times 10^5$, rabbit thyroglobulin (TG) $7{\cdot}74 \times 10^5$, human IgG (IgG) $1{\cdot}65 \times 10^5$, and bovine serum albumin (BSA) $0{\cdot}70 \times 10^5$.

SEA-X and SEA-X present in whole plasma. Similar results have been repeatedly observed with other plasma pools and other anti-X autoantibodies.

The recovery of SEA-HB upon molecular exclusion chromatography of mouse plasma was assessed by quantitative inhibition of anti-HB anti-erythrocyte autoantibodies (Fig. 6c). SEA-HB activity was recovered only in fraction A, the exclusion peak, and the character of the absorption profile was comparable to that observed with whole plasma. Autoantigen present in fraction A was antigenically complete and indistinguishable from plasma SEA-HB. Similar results have been repeatedly observed with plasma SEA-HB and anti-HB autoantibodies from a number of NZB mice.

DISCUSSION

Two independent anti-erythrocyte autoantibody responses in NZB mice have been described (Linder & Edgington, 1972a); one autoantibody reacts with exposed (X) erythrocyte surface antigens, the other with a cryptic (HB) antigenic site which can be exposed by treatment with proteolytic enzymes such as bromelin. The pathogenic significance of these autoantibody responses is indicated by the binding of both of these autoantibodies in association with the autoimmune haemolytic anaemia of NZB mice The presence of soluble analogues of these erythrocyte surface autoantigens has been suggested by the observation that anti-erythrocyte autoantibodies in NZB mice can be neutralized by plasmas from various mouse strains (Linder & Edgington, 1971a). The erythrocyte auto-antigens of NZB mice thus seem to be present in a soluble form in the plasma similar to the Lewis

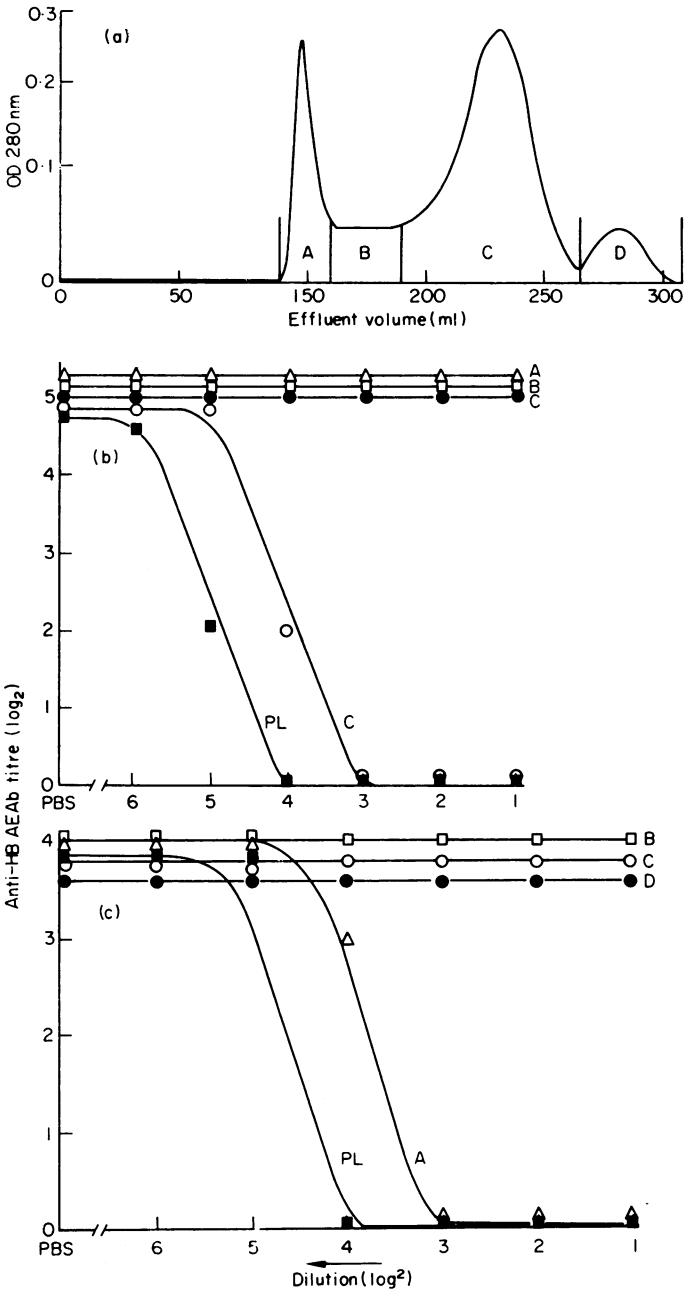

FIG. 6. Comparative inhibition of anti-X and anti-HB anti-erythrocyte autoantibodies by mouse plasma fractions. (a) Four fractions labelled A, B. C, and D were obtained by molecular exclusion chromatography on Biogel A-5. (b) Comparative inhibition of anti-X autoantibodies by plasma and each of the four plasma fractions. Complete inhibition was observed with plasma and fraction C, and these gave parallel absorption curves. Fractions A, B, and D had no inhibiting effect. (c) Comparative inhibition of anti-HB autoantibodies by plasma and each of the four plasma fractions. Complete and similar absorption was observed with plasma and fraction A; no inhibition was noted with the other plasma fractions.

antigen in humans (Marcus & Cass, 1969) and various blood group substances in other species (Stone, 1962; Andresen, 1962; Rasmussen, 1962). The presence of soluble analogues of cell surface antigens, capable of neutralization of cellular immunity, is also indicated by the recent results of Brawn (1971) who showed that lymphocytes sensitized to foreign histocompatibility antigens could be neutralized by serum only from mice of the histocompatibility type to which the lymphocytes had been sensitized.

The present results demonstrate that the two soluble erythrocyte autoantigenic activities present in plasma of mice can be separated by molecular exclusion chromatography. A high molecular weight fraction specifically neutralizes anti-HB anti-erythrocyte autoantibodies thus indicating the presence of SEA-HB. A smaller molecular weight fraction neutralizes anti-X autoantibodies and appears to contain SEA-X. Quantitative inhibition studies of anti-X and anti-HB autoantibodies indicate that the erythrocyte surface autoantigens differ from the soluble analogues with respect to concentration and frequently with regard to binding affinity of individual autoantibodies. Heterogeneity within individual NZB anti-X autoantibodies was indicated by the occasional observation of complex competitive inhibition slopes. These observations emphasize the similarity between the anti-erythrocyte autoantibody response of NZB mice and conventional heterimmune responses involving multiple clones of immunocompetent B lymphoid cells with consequent production of heterogeneous antibody populations (Eisen & Siskind, 1964). Our results would also appear to complement the previously observed heterogeneity of NZB anti-erythrocyte autoantibodies with regard to immunoglobulin class (Warner & Wister, 1968).

The simultaneous presence of antigen and antibody in the vascular compartment may permit the formation of circulating autologous immune complexes and contribute to the pathogenesis of glomerular lesions due to deposition of these complexes (Edgington, Glassock & Dixon, 1967; Dixon, Edgington & Lambert, 1967). The deposition of anti-HB autoantibodies, presumably in association with SEA-HB, in the glomeruli of aged mice with intercapillary glomerulosclerosis suggests a pathogenetically significant role for this autologous–immune complex system (Linder, Pasternack & Edgington, 1972). Preliminary data now indicate that anti-erythrocyte autoantibodies can be eluted from the glomerulonephritic kidneys of NZB mice (Linder & Edgington, 1972b).

The presence of independent anti-erythrocyte autoimmune responses to exposed (X) and hidden (HB) erythrocyte autoantigens may explain some of the contradictory results obtained in attempts to transfer autoimmune haemolytic anaemia from NZB mice to normal mice of different strains; successful transfer of autoimmune haemolytic anaemia with cell free filtrates of spleens from old NZB mice exhibiting characteristic autoimmune disease to five out of twenty-nine Swiss mice was suggested by the results obtained by Mellors & Huang (1967). However, similar but unsuccessful transfer experiments have been reported by Russell *et al.* (1970). The common observation in these transfer experiments was the absence of positive direct Coombs' test in the recipients. No anti-erythrocyte autoantibodies were demonstrated in sera of recipients by enzyme-augmented haemagglutination by Russell *et al.* (1970). Mellors & Huang (1967), however, were able to demonstrate anti-erythrocyte autoantibodies in recipients by a modified indirect Coombs' test employing enzyme-treated mouse erythrocytes as target cells. This modification of the enzyme-augmented haemagglutination assay is a very sensitive indicator for antibodies directed against certain hidden erythrocyte antigens including the HB autoantigen (Linder & Edgington, unpublished results). As such autoantibodies do not independently bind to erythrocytes *in vivo*—their

pathogenetic significance with respect to the autoimmune haemolytic anaemia is questionable (Linder & Edgington, 1972a) Similar anti-erythrocyte autoantibodies have been observed in C57Bl mice (Linder, Pasternack & Edgington, 1972) and in other mouse strains (Linder & Edgington, unpublished results) with increasing age in the absence of a positive direct Coombs' test. It must also be noted that cell free extracts of NZB spleens contain anti-X anti-erythrocyte autoantibodies apparently derived from plasma cells (Linder & Edgington, unpublished results). Transfer of these autoantibodies may only produce a transient positive direct Coombs' reaction, but may also stimulate the endogenous anti-HB anti-erythrocyte autoantibody response by anti-X autoantibody mediated exposure of hidden erythrocyte surface HB antigens.

The anti-X autoantibody response, which is characterized by positive Coombs' antiglobulin reactions, appears to be restricted to the NZB strain (Howie & Helyer, 1968) by genetic factors (Barnes *et al.*, 1972;) whereas we have observed the anti-HB autoantibody response more widely among mice (Linder, Pasternack & Edgington, 1972). Observed differences in the competitive inhibition slopes of individual autoantibodies suggest differing affinity for specific erythrocyte surface autoantigens. Differences in competitive inhibition of individual autoantibodies by SEA as contrasted with erythrocyte surface autoantigen suggest specificity for differing determinants on the autoantigen molecule. Thus, neither the anti-HB not the anti-X responses by B lymphoid cells appear clonally restricted and homogeneous in a fashion precise and characteristic for the NZB strain of mice. One previously considered hypothetical possibility, that there was genetic endowment of the NZB strain with a specific clone of autoimmunocompetent lymphoid cells to each given autoantigen, is not supported by our data. If genetic factors responsible for these responses in the NZB strain operate at the level of primary endowment with B lymphoid cell clones, it would have to be postulated that even to a single autoantigen multiple clones of autoimmunocompetent cells are genetically provided. The observed variations in affinity for and competitive binding of SEA-X or SEA-HB by specific anti-erythrocyte autoantibody responses of individual mice indicate proliferation and differentiation of a variety of different clones of anti-X and anti-HB autoimmunocompetent B cells in different individual NZB mice.

It appears well established that overall immunological tolerance may be induced and maintained with low antigen dose at the T cell level and with high antigen dose at the B cell level (Weigle, 1971). In this study we demonstrated distinct differences in antigenic determinant expression between the cell surface bound and the soluble forms of two erythrocyte autoantigens. Hypothetically, tolerance may be established to one form of the molecule and the other may function as immunogen and recruit autoimmunocompetent B lymphocyte clones directly, or through T cell co-operation, to differentiate and synthesize antibody in violation of an unresponsive state (Benjamin & Wiegle, 1970). In addition, recent work has suggested B cell suppressive functions for T lymphocytes (Gershon *et al.*, 1972), aberrations of which might play a significant role in B lymphocyte autoimmunity. In view of the existence of both B cell-independent immune responses and T cell-dependent immune responses, three conceptual approaches to these genetically presupposed autoantibody responses to a specific molecule such as the murine erythrocyte autoantigens appear attractive and could accommodate the observations.

First, T cell suppression of autoimmunocompetent B lymphocytes, normally present in mice, may be defective. Either on a qualitative or quantitative basis, T suppressor cells may

be unable to mitigate spontaneous randon B cell activation, derepression associated with other cellular events such as viral infection, or direct immunogen induced activation of these cells. Second, the quantitative degree of B cell repression, required for normal immunological homeostasis, may be reduced in NZB mice, thus permitting more ready derepression by what should represent subthreshold stimulation. Autoantigens, of moderate replicate determinant density, usually below the threshold of independent B lymphocyte recruitment (Feldman & Basten, 1971), could then exceed an abnormally low threshold of stimulation required for B lymphocyte depression. Similarly, other events such as viral infection or random derepression might be inadequately suppressed. Third, aberrations of immunological homeostasis may occur through abuse of cellular co-operation much as suggested by a number of investigators (Allison, Denman & Barnes, 1971; Weigle, 1971). Induction of the anti-erythrocyte autoantibody responses would then follow immunological recognition by T cells of an additional determinant(s) on the autoantigen molecules such as acquisition of neoantigen expression through molecular reconformation (Weigle, 1965; Plow & Edgington, 1972) or attachment to other antigenic moieties including virus (Allison, Denman & Barnes, 1971). Alternatively, one could also postulate that these mice are genetically endowed with T cells capable of recognizing a specific determinant(s) on the autoantigen and thus mediate co-operative recruitment of a variety of normally occurring autoimmunocompetent B lymphocytes.

All of these hypothesis presume major control of autoimmunological homeostasis by T cell function and the presence of immunologically competent B lymphocytes with specificity for a variety of determinants for given autoantigen molecules. Presumably the low concentrations of soluble erythrocyte autoantigens in body fluids are inadequate for the induction and maintanence of B cell tolerance, and the status of T cell tolerance to these autoantigens is not known. Our data supports the genesis of anti-erythrocyte autoimmune responses at levels of biological organization beyond genetic endowment with specific B lymphoid cell clones, and reasserts hypothetically proposed concepts for the genesis of B cell autoimmunity at the level of control and derepression of autoimmunocompetent B lymphocytes normally present in the host.

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