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Hyperactivity of donor B cells after neonatal induction of lymphoid chimerism in mice

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SUMMARY

Balb/c mice made chimaeric by neonatal injection of semi-allogeneic (A/J × Balb/c)F1 hybrid spleen cells develop anti-DNA and rheumatoid factor-like antibodies in the context of hypergammaglobulinaemia with marked elevation of IgG1 and IgE serum levels. Chimaeric mice also display increased levels of antibodies to different haptens and to tobacco mosaic virus (TMV). The allotypic marker of the A/J strain is present on anti-DNA and anti-hapten antibodies. In addition, spleen cells of chimaeric mice spontaneously produce high levels of IgG1 and anti-DNA antibodies *in vitro* and this hyperactivity is abolished after lysis of donor lymphocytes. These findings indicate that polyclonal activation of donor B cells plays an important role in this model of autoimmunity.

Keywords Neonatal allotolerance Autoimmunity B-cell activation

INTRODUCTION

The induction of tolerance to alloantigens by neonatal injection of semi-allogeneic spleen cells in mice results in an autoimmune syndrome characterized by high levels of anti-DNA antibodies and immune complex-mediated glomerulonephritis (Goldman *et al.*, 1983; Tateno *et al.*, 1985; Luzuy *et al.*, 1986). Lymphoid chimerism is required for the occurrence of autoimmunity and recent studies indicate that F1 donor B cells injected at birth are responsible for the production of anti-DNA and of nephritogenic antibodies (Luzuy *et al.*, 1986; Abramowicz *et al.*, 1987b). There is evidence that these cells are activated by a subset of host T cells which escape tolerance induction (Abramowicz *et al.*, 1987a; Merino *et al.*, 1987). However, the mechanisms leading to autoimmunity in this situation are not well defined. In this respect, it is of interest to determine whether the autoantibodies occur in the context of polyclonal B-cell activation. Indeed, polyclonal B-cell activation appears as a major pathogenic mechanism in several other models of systemic autoimmunity including spontaneous lupus-like diseases (Klinman & Steinberg, 1987) and autoimmune syndromes induced by infectious or toxic agents (Fournie *et al.*, 1983).

The aim of this study was to analyse the isotypic profile of circulating immunoglobulins in Balb/c mice injected at birth with (A/J × Balb/c) F1 hybrid spleen cells and to compare the levels of autoantibodies with the levels of anti-hapten and anti-viral antibodies. The involvement of F1 donor B cells in the observed abnormalities was evaluated by the allotypic characterization of anti-DNA and anti-hapten antibodies. In addition, we determined the effect of the lysis of donor lymphocytes on the *in vitro* production of antibodies by spleen cells of chimaeric mice.

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MATERIALS AND METHODS

Mice. The inbred strains A/J (H-2^k), Balb/c (H-2^d) and C57Bl/6 (H-2^b) were purchased from Olac (Bicester, UK). The (A/J × Balb/c) and (C57Bl/6 × Balb/c) F1 hybrids were bred at our own colony.

Experimental protocol. Chimaeric animals were obtained by intraperitoneal inoculation of newborn Balb/c mice (less than 24 h after birth) with 10×10^7 spleen cells from (A/J × Balb/c) F1 hybrids. Controls animals consisted of Balb/c mice neonatally injected with 10×10^7 syngeneic spleen cells. In some experiments, newborn Balb/c mice were intravenously injected with 3×10^7 T-cell-depleted full allogeneic A/J spleen cells, as previously described (Abramowicz *et al.*, 1987b). Since the peak levels of serum IgG and anti-DNA antibodies occur between 3 and 5 weeks after neonatal injection of semi-allogeneic spleen cells (Goldman *et al.*, 1983; Luzuy *et al.*, 1986), 4-week-old mice were used in the present study.

Determination of serum immunoglobulin levels. Serum levels of IgG1, IgG2a, IgG2b, IgG3, IgA, IgM and IgE were measured by solid-phase enzyme-linked immunosorbent assay (ELISA) using monospecific antisera obtained from Sigma Chemical Co

(St Louis, MO, USA) except for rabbit anti-mouse IgE antiserum purchased from Miab (Uppsala, Sweden). Briefly, microtitre plates (Nunc, Rockswild, Denmark) were coated with the IgG fraction of each antiserum in 0·05 M carbonate buffer and then saturated with 1% bovine serum albumin (BSA) in phosphate-buffered saline. Appropriate dilutions of serum samples were incubated in the plates for 2 h at room temperature. After washing, bound antibodies were revealed by corresponding anti-serum conjugated with alkaline phosphatase or biotin. The specificity of each assay was established by the lack of detection of monoclonal antibodies bearing other isotypes than the one tested. The values of individual serum samples were referred to a standard curve made with a pool of sera from 4-week-old normal Balb/c mice.

Detection of anti-DNA and rheumatoid factor antibodies. Anti- single-stranded DNA and anti-rabbit IgG activities were evaluated by solid-phase ELISA as previously described (Abramowicz *et al.*, 1987b). Serial dilutions of the serum samples were incubated in microtitre plates coated with either single-stranded DNA prepared from calf thymus DNA (Sigma Chemical Co, St Louis, MO, USA) (Pisetsky & Peters, 1981) or IgG purified from a normal rabbit serum. After washing, alkaline phosphatase-conjugated goat anti-mouse IgG or IgM antiserum was used to reveal bound antibodies. Results were expressed as titration units by reference with a standard serum.

Anti-hapten and anti-viral antibodies. IgG and IgM antibodies to arsonate (ARS), fluorescein isothiocyanate (FITC), trinitrophenyl (TNP) and tobacco mosaic virus (TMV) were determined by ELISA using the same procedure as for anti-DNA and rheumatoid factor antibodies. In the anti-hapten assays, plates were coated with ARS-BSA, FITC-BSA or TNP-BSA conjugates (4 μg/ml in carbonate buffer), while an antigenic extract of TMV (Jeener, 1965) was used for determination of anti-TMV antibodies.

The possible cross-reaction of anti-DNA antibodies with TNP was evaluated in competition experiments in which a pool of sera from experimental mice was incubated with DNA or TNP-BSA prior to testing for anti-TNP or anti-DNA activity (Klinman & Steinberg, 1987).

Detection of donor allotype by ELISA. A polyclonal antiserum to the Ig^e allotype expressed on IgG2b molecules of the A/J strain was raised in Balb/c mice, as previously described (Abramowicz *et al.*, 1987b). The affinity-purified IgG fraction of this antiserum was conjugated to biotin and used as revealing agent in the anti-DNA or anti-TNP ELISA to detect antibodies produced by (A/J × Balb/c) F1 or A/J donor B cells. Antibodies produced by host Balb/c or (A/J × Balb/c) F1 donors B cells were revealed by anti-Ig^a allotype antibodies raised in A/J mice.

In order to quantify the levels the levels of Ig^e allotype, samples were incubated in wells coated with goat anti-mouse IgG2b antiserum before addition of a biotinylated monoclonal anti-allotype antibody. Results were calculated by reference with a standard curve made with IgG chromatographically purified from normal A/J mouse serum of known IgG2b concentration.

Spontaneous secretion of antibodies by spleen cells in culture. Spleen cells (10×10^6/ml) were cultured in medium RPMI 1640 supplemented with 5% fetal calf serum, 1% pyruvate, 1% glutamine, 1% non-essential amino-acids, 100 U/ml of penicillin, 100 mg/ml of streptomycin and 5×10^{-5} M 2-mercaptoethanol. After 2 days of incubation at 37°C in 6% CO_2 in humidified air, supernatants of the cultures were tested by ELISA for IgG1, anti-DNA and Ig^e-bearing antibodies, as described above.

Table 1. Immunoglobulin levels in 4-week-old chimaeric mice

Mice	Serum levels* of IgG_1	IgG_{2a}	IgG_{2b}	IgG_3	IgA	IgM	IgE
Controls	77 ± 12	106 ± 11	102 ± 19	102 ± 6	102 ± 24	104 ± 7	86 ± 16
($n=11$)							
Chimaeric†	1,911‡ ± 263	303‡ ± 24	177‡ ± 17	794‡ ± 158	41 ± 6	275‡ ± 41	9,832‡ ± 2,734
($n=10$)							

* Expressed as percentage of the value of a normal mouse serum.
† Chimaeric mice were obtained by injection of 10^8 spleen cells from (A/J × Balb/c) F_1 adult mice into Balb/c newborns.
‡ $P < 0.01$ as compared with control mice.

In vitro lysis of donor lymphocytes. Three monoclonal cytolytic anti- $H2^k$ antibodies (Tib 92, Tib 93 and HB 16) purchased from the American Type Culture Collection were used to evaluate the involvement of donor lymphocytes in the *in vitro* production of immunoglobulins by spleen cells of Balb/c mice neonatally injected with (A/J × Balb/c) F1 cells. Spleen cells of chimaeric mice were incubated at a concentration of 3×10^7/ml in RPMI 1640 with appropriate dilutions of the anti-$H2^k$ antibodies for 45 min at 4°C. Cells were then pelleted and incubated at the same concentration in a 1:10 dilution of baby rabbit complement (Cederlane Laboratories. Ontario, Canada) before being tested for remaining antibody secretion. As control of specificity, we determined the effect of the treatment with anti-H-2^k antibodies on the hyperactivity of spleen cells from Balb/c mice neonatally injected with 10^8 (C57B1/6 × Balb/c) (H-$2^{b/d}$) F1 spleen cells.

Statistical analysis. Comparison between groups of mice was made by Wilcoxon's rank sum test.

RESULTS

Hyperimmunoglobulinaemia in chimaeric mice. As shown in Table 1, serum levels of IgG1, IgG2a, IgG2b, IgG3, IgM and IgE were significantly higher in mice injected with (A/J × Balb/c) spleen cells than in control mice. A marked increase was observed for IgG1 among the IgG subclasses, and a salient feature was the very high levels of IgE which were about 100-fold above control values (Table 1).

Antibody activities in serum of chimaeric mice. We first confirmed that chimaeric mice produce high titres of IgG anti-DNA, serum levels being approximately 30 times higher than in control mice ($P < 0.01$). Rheumatoid factor-like IgG antibodies were also present but at a lower titre ($P < 0.01$) (Fig. 1a). In order to determine whether these autoantibodies occur in the context of polyclonal B-cell activation, antibody activities to TMV and to different haptens were measured. As shown in Fig. 1a, chimaeric mice exhibited higher serum IgG levels to TMV, FITC-BSA, TNP-BSA and ARS-BSA than control mice

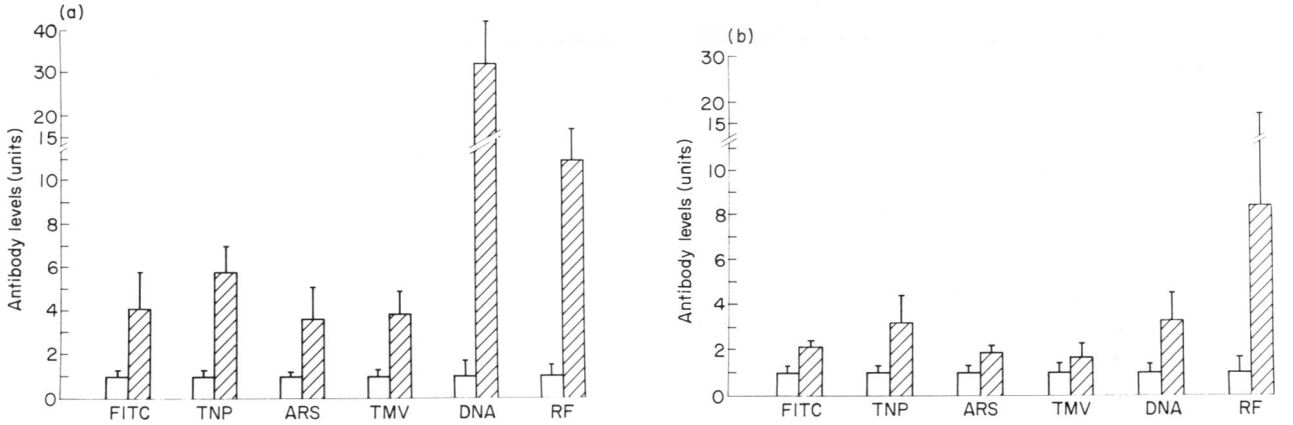

Fig. 1. Serum levels of antibodies to FITC, TNP, ARS, TMV, DNA and rabbit IgG in 4-week-old chimaeric mice (hatched columns) and in age-matched controls (open columns). IgG (Fig. 1a) and IgM (Fig. 1b) activities to these antigens are expressed as titration units. the mean values + s.e.m. of eight to 12 mice are represented.

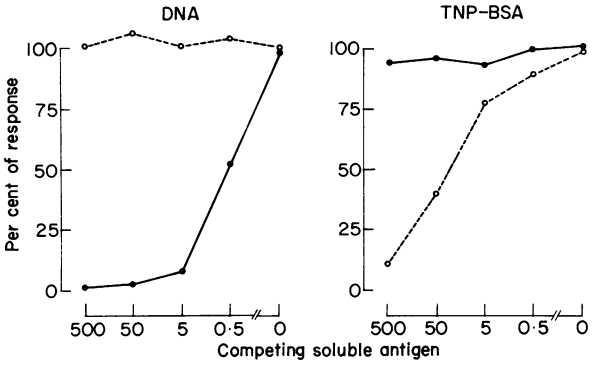

Fig. 2. A pool of sera from chimaeric mice was assayed for (●) anti-DNA and (○) anti-TNP IgG antibodies in the presence of various concentrations of DNA or TNP-BSA as competing antigen. Only when the competing antigen was identical to the antigen coated on the plate was there a significant inhibition of the ELISA signal.

Table 2. Allotypic characterization of anti-DNA antibodies in chimaeric mice

	No. of mice		
		With anti-DNA antibodies bearing	
Neonatal inoculum	Total	Balb/c allotype*	A/J allotype*
(A/J × Balb/c) F_1 spleen cells	9	8	9
A/J T cell-depleted spleen cells	8	1	7

* Anti-DNA antibodies bearing the Ig^a allotype of the Balb/c strain or Ig^e allotype of the A/J strain were detected by ELISA. Serum was considered as positive if its signal exceeded the mean value of controls + 3 s.d.

($P < 0{\cdot}01$ for each assay), whereas no significant activity was detected to unconjugated BSA (data not shown). The anti-hapten antibodies were present at lower titres than autoantibodies but IgG anti-TNP levels were still about six times higher than those of control mice. Inhibition experiments presented in Fig. 2 indicated that the anti-TNP activity could not be attributed to cross-reacting anti-DNA antibodies (Serban *et al.*, 1985) since it was not inhibited by DNA whereas the anti-DNA activity was efficiently inhibited by DNA but not by TNP.

IgM activities to DNA, rabbit IgG, TMV and to the different haptens were also elevated in chimaeric mice ($P < 0{\cdot}01$ for each test) but the levels of IgM anti-DNA were much lower than those of IgG anti-DNA (Fig. 1b).

Donor B cells produce anti-DNA and anti-hapten antibodies. In serum of Balb/c mice neonatally injected with (A/J × Balb/c) cells, Ig^a- as well as Ig^e-bearing anti-DNA antibodies were detected (Table 2). Similarly, the Ig^e allotype was detected on anti-TNP antibodies in nine out of 13 mice. This indicated that donor B cells were involved in the production of anti-DNA and of anti-hapten antibodies.

In order to analyse the possible participation of host B cells in the autoimmune syndrome, some animals were inoculated with full allogeneic T-cell-depleted A/J spleen cells. Although the T-cell depletion required to prevent lethal graft *vs* host reaction attenuated the autoimmune syndrome (Tateno *et al.*, 1985; Abramowicz *et al.*, 1987b), anti-DNA antibodies were still detected in these animals. The presence of A/J donor allotype (Ig^e) together with the absence of Balb/c host allotype (Ig^a) on anti-DNA antibodies in seven out of eight mice (Table 2) indicated that donor B cells played a dominant role in the anti-DNA response.

Enhanced production of antibodies by spleen cells in vitro. As shown in Table 3, spleen cells from Balb/c mice injected at birth with (A/J × Balb/c) F1 cells spontaneously produce high levels of IgG1 and anti-DNA *in vitro*. The secretion of these antibodies as well as the presence of the Ig^e donor allotype in the culture supernatants were abrogated when the donor lymphocytes were lysed with appropriate anti-H-2^k antibodies prior to the spleen cell culture. The specificity of these findings was demonstrated by the inability of the anti-H-2^k antibodies to suppress the hyperactivity of spleen cells from Balb/c mice injected with (C57Bl/6 × Balb/c) F1 cells (Table 3).

Table 3. Spontaneous production of antibodies by spleen cells

Neonatal inoculum*	Treatment of spleen cells†	*In vitro* production of‡ IgG1 (ng/ml)	anti-DNA (OD 405 nm)	Ig^e allotype (ng/ml)
Balb/c ($n=4$)	C★	82±52§	221±63	ND¶
A/J × Balb/c ($n=4$)	C★	760±179‖	1,165±386‖	8·8±3·9
	anti-H-2^k and C	84±46**	130±20**	<1·8**
C57Bl/6 × Balb/c ($n=2$)	C★	538	881	ND
	anti-H-2^k and C★	485	746	

* 10^8 spleen cells were injected intraperitoneally into Balb/c newborns.

† Spleen cells (5×10^6/ml) from 6-week-old mice were incubated with complement (C★) alone or with anti-H-2^k antibodies followed by C★.

‡ Supernatants were collected after 2 days of culture.

§ Mean ± s.e.m.

¶ ND: not done.

‖ $P<0{\cdot}05$ as compared with Balb/c controls; ** $P<0{\cdot}05$ as compared with C★ alone.

DISCUSSION

The data presented in this paper first indicate that hyperimmunoglobulinaemia in mice made chimaeric by neonatal injection of semi-allogeneic spleen cells results from polyclonal activation of donor B-cells. Features of polyclonal B-cell activation include increased serum levels of anti-hapten, anti-TMV, anti-DNA and rheumatoid factor-like antibodies as well as the spontaneous production of high levels of IgG1 and anti-DNA antibodies by spleen cells *in vitro*. The involvement of donor B cells in this activation is demonstrated by the presence of donor-specific allotype on circulating anti-DNA and anti-hapten antibodies. *In vitro* experiments further indicate that donor B cells are responsible for the increased production of immunoglobulins since lysis of donor cells abrogates the enhanced production of antibodies by spleen cells of chimaeric mice. It is possible that the elimination of donor T cells contributes to this suppression of B-cell hyperactivity *in vitro* since we previously observed that the presence of T cells in the neonatal inoculum was required for the development of hypergammaglobulinaemia *in vivo* (Abramowicz *et al.*, 1987b). Our data confirm those of Luzuy and co-workers who demonstrated using allotype-congenic mice in the (C57Bl/6 × Balb/c) F1—> Balb/c strain combination that donor B cells represent the only source of autoantibodies in chimaeric animals (Luzuy *et al.*, 1986). Interestingly, hypergammaglobulinaemia with predominance of IgG1 and activation of donor B cells have also been described in a completely different strain combination in which repeated injections of semi-allogeneic cells in the early days of life induce fatal host *vs* graft disease (Hard, 1980; Hard, Carter & Bick, 1983). Thus, donor B-cell hyperactivity after neonatal injection of semi-allogeneic spleen cells does not appear to be restricted to a particular strain combination.

Comparison of serum titres of anti-hapten antibodies with those of autoantibodies clearly demonstrates that there is a preferential stimulation of IgG anti-DNA antibodies in chimaeric mice. Similar observations made in mice undergoing the stimulatory form of chronic graft *vs* host (GVH) disease (Gleichmann *et al.*, 1984) led to hypotheses that the repetitive epitopes of DNA provide an additional stimulatory signal to those alloactivated B cells which are committed to produce anti-DNA antibodies (Gleichmann, Van Elven & Van der Veen, 1982). In mice with neonatal chimerism, it is also possible that Ly-1+B cells which are known to be a major source of autoantibodies are preferentially expanded after their inoculation in the newborn host, as reported after transfer of peritoneal cells from allotype-congenic mice (Forster & Rajewsky, 1987).

The isotypic profile of the antibodies produced in this model is quite different from that induced by classical B-cell mitogens such as bacterial endotoxins (Izui. Eisenberg & Dixon, 1981). In chimaeric mice, there is a preferential production of IgG1 and IgE antibodies. The secretion of both isotypes is known to require T-cell-derived signals (Bankhurst, Lambert & Miescher, 1975; Katz, 1980), and a tremendous increase of serum IgE has been reported in rats with mercury-induced autoimmune syndrome, a model of T-cell-dependent polyclonal B-cell activation (Hirsch *et al.*, 1982). This supports the hypothesis that host T cells are responsible for donor B-cell activation in chimaeric mice (Luzuy *et al.*, 1986). Indeed, although the neonatal inoculation of semi-allogeneic spleen cells efficiently induces tolerance to the injected alloantigens as indicated by the acceptance of skin grafts (Billingham & Brent, 1959) and the abolition of proliferative and cytolytic T-cell responses (Brent *et al.*, 1976), a subset of host T cells appears to remain able to stimulate donor B cells both *in vivo* (Merino *et al.*, 1987) and *in vitro* (Abramowicz *et al.*, 1987a). Thus, allogeneic T-B interactions similar to those occurring during stimulatory GVH reaction (Gleichmann *et al.*, 1984) are probably operative in this model. The mediators of these interactions remain to be characterized. However, it has been recently shown that B-cell activation in mice undergoing chronic GVH disease is associated with the production of a B-cell differentiation factor (B151-TRF2) which is able to induce autoantibody secretion (Dobashi *et al.*, 1987). In mice made neonatally chimaeric, the preferential induction of IgG1 and IgE antibodies suggests that interleukin 4 could also be involved in the process of B-cell activation (Paul & Ohara, 1987).

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