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Immunology

Disappearance and reappearance of B cells after *in vivo* treatment with monoclonal anti-I-A antibodies

(immunotherapy/B-cell subpopulations/isotype regulation/fluorescence-activated cell sorting)

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ABSTRACT **Previous studies have shown that treatment with antibodies to the murine I-A antigen encoded in the major histocompatibility complex attenuates experimental allergic encephalitis and experimental autoimmune myasthenia gravis. These studies were conducted with SJL mice, an inbred strain that is highly susceptible to the induction of these diseases. Here we show that injection of monoclonal anti-I-A antibody in the amounts used for the above studies rapidly depletes B cells. Fluorescence-activated cell sorter (FACS) multiparameter analysis of the B-cell subpopulations in treated animals shows that maximum depletion occurs around 5 days after treatment and that recovery of some subpopulations is still incomplete 1 month later. SJL mice are more sensitive to this B-cell depletion and recover more slowly than putatively normal C3H.Igh^b (CKB) mice. Some components of the primary, secondary and tertiary IgG antibody responses are reduced in anti-I-A-treated SJL animals immunized after the first and second anti-I-A injections. The persistence of some antibody response impairment well beyond the time when anti-I-A disappears raises a note of caution concerning human therapy protocols based on the injection of anti-Ia antibodies.**

Injecting adult mice with monoclonal antibodies against I-A antigen interferes with helper T-cell activation for antibody responses (1) and contributes to the induction of suppressor T cells that reduce delayed-type hypersensitivity responses (2). In addition, such anti-I-A treatment sharply curtails antibody production to antigens that elicit responses under immune response (*Ir*) gene control (3) and attenuates two murine autoimmune diseases inducible in SJL mice: experimental autoimmune encephalitis (EAE) and experimental autoimmune myasthenia gravis (EAMG) (4, 5). These experiments open a new approach to the treatment of human autoimmune disease (6). However, we describe here severe and prolonged depletions of splenic and lymph-node B cells and impaired antibody responses induced by anti-I-A injections. These findings may limit the universal application of anti-I-A therapy.

Repeated injections of 0.6–1.8 mg/week of monoclonal anti-I-A antibody starting in the neonatal period markedly reduces the number of IgM-bearing B cells in spleens of mice up to age 7 weeks (7). Here we show that even a single injection of 4 mg of monoclonal anti-I-A antibody into an adult SJL/J mouse is sufficient to virtually eliminate B cells within 5 days and that subsequent recovery is slow. Complex effects on antibody responses are also presented below.

B cells were recently subdivided into three major subpopulations based on correlated IgM and IgD levels, using multiparameter measurements made with a fluorescence activated cell sorter (FACS) (8, 9). Here we have followed the B-cell depletion and recovery phases induced by anti-I-A treatment by similar FACS subpopulation analyses in SJL and in a second strain, C3H.Igh^b (CKB). CKB cells provided the immunogen for generating the 10-3.6 hybridoma, which makes the anti-I-A used in these studies. It also has the same immunoglobulin allotype as SJL but does not have the immunologic abnormalities seen in SJL (10, 11).

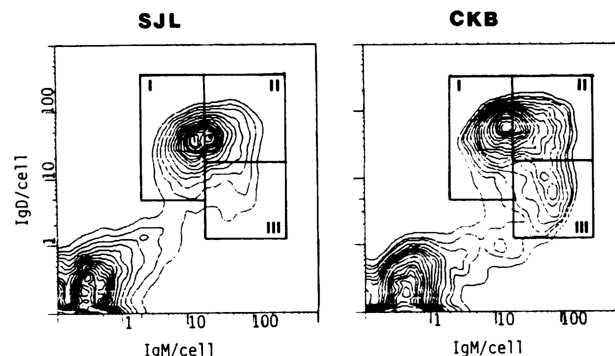

FIG. 1. Splenic B cell subpopulations I, II, and III in adult SJL/J and CKB mice. Spleen cells were stained with fluorescein anti-IgM together with biotin anti-*Igh-5^b* (IgD b allotype) followed by Texas red avidin and analyzed on a dual-laser FACS equipped with logarithmic amplifiers. Axes represent the relative amounts of the respective antigens as revealed by fluorescence intensity.

MATERIALS AND METHODS

Animals. Young adult SJL/J mice were obtained from our colony, and CKB (C3H.Igh^b) mice were provided by H. McDevitt.

Monoclonal Antibodies. The anti-I-A antibody was from the hybridoma 10-3.6. It recognizes the public specificity Ia.17 associated with $H\text{-}2^{k,r,s,f}$ and is *Igh-1^b* allotype (12). It was used as ascites fluid or purified by ammonium sulfate precipitation and acid elution from staphylococcal protein A-Sepharose. Rat anti-mouse IgM (13), mouse anti-mouse *Igh-5^b* (IgD b allotype) (14) and mouse anti-*Igh-1^b* (IgG2a) (15) were purified and fluorochrome-coupled as described (16).

Two-Color Immunofluorescence Analyses. Preparation of cells, staining procedures, and data collection procedures were as described (16). Large-angle scatter (near 90 degrees) was used to assess cell granularity. Cells falling within the macrophage range of large-angle scatter were not examined in our analyses. Two-color staining data are presented as "contour plots" (Figs. 1 and 2) that are representations of three-dimensional surfaces in which the levels of green and red fluorescence per cell define locations on a two-dimensional surface, and the frequency of cells with that value of

Abbreviations: EAE, experimental autoimmune encephalitis; EAMG, experimental autoimmune myasthenia gravis; FACS, fluorescence-activated cell sorter; KLH, keyhole limpet hemocyanin; CGG, chicken gamma globulin; DNP, 2,4 dinitrophenyl hapten; Ir, immune response.

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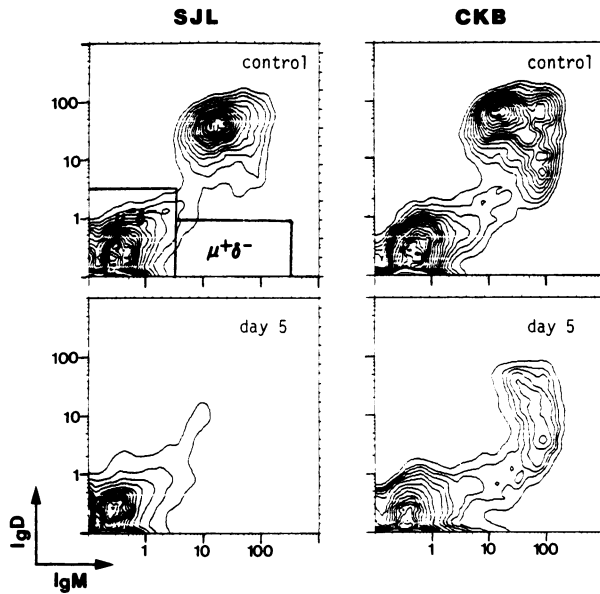

FIG. 2. Depletion of splenic B cells in adult SJL/J and CKB mice 5 days after a single injection of 4 mg of anti-I-A antibody. The integration boundaries used to determine the frequency of IgM$^+$, IgD$^-$ and IgM$^-$, IgD$^-$ cells (autofluorescence background) are marked on the plot.

fluorescence defines the elevation at that location. After this surface is smoothed, contour lines are drawn to divide the sample into equal fractions.

Measurement of Serum Anti-I-A Antibody. Dilutions of serum of treated animals were incubated with 5×10^5 SJL/J spleen cells for 30 min at 0°C and then washed with staining medium. Fluoroscein-conjugated anti-*Igh-1^b* was used to detect anti-I-A bound to cells. The sensitivity of this assay is 0.2 μg/ml of anti-I-A. A standard curve was made by using purified anti-I-A monoclonal 10-3.6 antibody.

RESULTS AND DISCUSSION

CKB and SJL mice show fairly similar splenic IgM/IgD subpopulation distributions—i.e., the predominant B-cell subpopulation (I) tends to be equivalent in frequency in the two strains, whereas the other splenic subpopulations (II and III) tend to be somewhat larger in CKB (Fig. 1). Although IgM$^-$, IgG$^+$ B cells play a key role in immunologic memory, the frequency of these cells is too low to measure as a splenic population by FACS. Therefore, we mainly contrasted the effects of anti-I-A treatment in SJL and CKB mice on the overall frequencies of IgM$^+$, IgD$^+$ B cells and IgM$^+$, IgD$^-$ B cells in the treated animals.

SJL mice are very sensitive to anti-I-A treatment. One day after these mice were treated with anti-I-A antibodies (0.5 ml of monoclonal 10-3.6 ascites fluid containing 4 mg of anti-I-A per mouse), nearly half of the splenic IgM$^+$, IgD$^+$ B cells

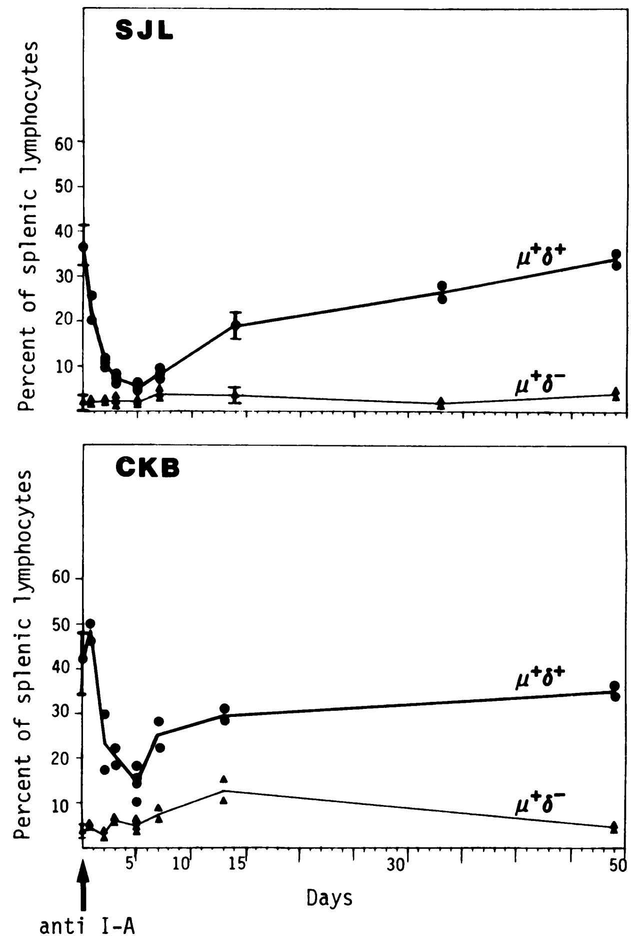

FIG. 3. Frequencies of splenic B cells that bear either IgM alone (μ^+, δ^-) or both IgM and IgD (μ^+, δ^+) as determined by two-color FACS analysis after a single injection of anti-I-A antibody. The frequency of IgM$^+$, IgD$^+$ cells was obtained by subtracting the frequency of IgM$^+$, IgD$^-$ cells from the frequency of cells above the autofluorescence background (as defined by the integration boundary for double-negative cells shown in Fig. 2). Means and standard deviations are given when there were more than six animals per group, otherwise individual animals are shown.

were missing. Four days later (on day 5), virtually all of the B cells were gone (Figs. 2 and 3). Recovery began shortly thereafter; however, although the splenic B-cell frequency rose to roughly normal levels within the next 3 weeks (by day 33), the predominant B-cell population in adult mice (labeled I in Fig. 1) was still well below its normal frequency. This population, which normally appears late in B-cell ontogeny (8), was also the last to reappear in the treated mice. Thus, 33 days after anti-I-A injection, population I contained roughly 20% of the spleen cells rather than the usual 30% of spleen cells found in untreated animals.

CKB mice show a similar but somewhat lower sensitivity

Table 1. Anti-I-A treatment depletes IgM$^+$, IgD$^+$ B cells from spleen and lymph node

Days after anti-I-A treatment	IgM$^+$, IgD$^+$ cells,* %			
	SJL/J mice		CKB mice	
	Spleen	Lymph node	Spleen	Lymph node
Untreated	37 ± 5	10 ± 3	42 ± 6	15 ± 3
2	11 (9, 12, 12)	3 (2, 3, 3)	24 (17, 30)	10 (10, 10)
5	5 ± 1	1 (1, 1)	14 ± 3	6 (5, 6)
14	18 ± 3	4 ± 2	36 (35, 36)	8 (7, 8)
49	34 (33, 34)	10 (10, 10)	36 (35, 36)	11 (10, 11)

*Determined as in the legend of Fig. 3. Means and individual mouse values are indicated unless four or more mice were tested, in which case the SD is given.

to anti-I-A (see Figs. 2 and 3). The overall B-cell depletion in spleen was maximal in both strains on day 5 but was never as complete in CKB as in SJL mice. Lymph node B cells showed roughly the same patterns as splenic B cells, both with respect to timing and to the extent of depletion (Table 1). In addition, CKB animals developed a significant population of IgM^+, IgD^- cells in spleen shortly after treatment, while SJL mice (like untreated animals of both strains) had relatively few of these cells.

Finally, CKB mice appear to lose their B cells somewhat more slowly than do SJL mice. That is, although anti-I-A levels declined at similar rates in the two strains (see below), SJL mice lost about half their B cells within 24 hr of treatment, whereas the two CKB animals tested at this time point actually had slightly increased numbers of splenic B cells. These findings with CKB mice agree with previous evidence (7) demonstrating that anti-I-A does not immediately (1 day after treatment) deplete B cells from adult C3H mice. However, our studies demonstrate that after 1 day, there is a profound B-cell depletion in CKB mice.

One 4-mg dose of anti-I-A, used for the above studies, is sufficient for maximal B-cell depletion. No further reduction in B-cell frequency was obtained by injecting a second dose two days later (Table 2). Furthermore, although we routinely injected 4 mg of anti-I-A, 1/10th that amount (0.4 mg) was still sufficient to reduce splenic B-cell frequencies by about 50% on day 5 (Table 2). Thus, the failure to completely eliminate B cells is not due to treatment with inadequate amounts of anti-I-A antibody.

Similarly, the long-term perturbation of B cell subpopulations (see Fig. 3) in anti-I-A-treated mice does not depend upon long-term persistence of injected antibody. That is, although anti-I-A levels in serum rose immediately after a single 4-mg injection of anti-I-A and were still above 1 mg/ml 17 hr later, they dropped during the next 2 days to about 0.01 mg/ml on day 3 and to less than 0.2 μg/ml by day 7.

The effects of anti-I-A treatment on antibody responses persist even longer than noticeable effects on B-cell frequencies. We followed the protocol used in earlier work demonstrating that anti-I-A treatment impairs *Ir* gene-controlled antibody responses to the synthetic polypeptide (H,G)-A–L (3). To develop a more general view of the effect of anti-I-A treatment on antibody responses, we immunized with typical hapten–carrier conjugates (responses to which are not under differential *Ir* gene control) and measured individual IgG isotype responses to the hapten and carrier after each immunization. Thus, we injected SJL mice with anti-I-A on the day before and the day after immunization with 100 μg (on alum) of dinitrophenyl (DNP)-conjugated keyhold limpet hemocyanin (KLH), repeated this treatment and immunization cycle 1 week later, and finally injected the animals with DNP on a second carrier protein, chicken gamma globulin (CGG) 8 weeks after the second treatment and immunization cycle.

Table 2. One 4-mg injection of anti-I-A antibody causes maximal B-cell depletion in SJL/J mice

Anti-I-A injected, mg			
Day 0	Day 2	Test day	Splenic IgM^+ cells,* %
—	—		39 ± 5
0.4†	—	5	23 (16, 29)
1.2†	—	5	19 (17, 21)
4†	—	5	8 ± 1
4†	—	7	12 ± 1
4†	4†	7	8 (9, 7)
4‡	4‡	7	11

*The percentage of IgM^+ cells was determined by subtracting IgM^-, IgD^- cells (using boundaries indicated in Fig. 2) from the total lymphocytes.
†Ascites containing indicated amount.
‡Purified antibody.

Data from these studies show clearly that anti-I-A treatment results in the selective, long-term impairment of components of the antibody response to priming antigens presented 1 day after the first anti-I-A injection (Tables 3 and 4). Virtually all treated animals showed reductions in primary, secondary, and tertiary responses; however, by and large, primary responses tended to be more impaired than secondary and tertiary responses, IgG1 responses tended to be more impaired than IgG2a and IgG2b responses, and anti-carrier responses tended to be more impaired than anti-hapten responses. Surprisingly, IgG2a and IgG2b anti-hapten responses in most of the treated animals reached essentially normal secondary levels after the second immunization, despite severe B-cell deficiencies in the immunized animals at the time they were restimulated (Tables 3 and 4).

In the third immunization in this series, the antigen (DNP-CGG) was introduced 8 weeks after the last anti-I-A injection. These animals no longer had detectable amounts of anti-I-A in circulation (data not shown) and had regained normal numbers of B cells (Fig. 3). Nevertheless, their IgG1 anti-DNP responses were significantly below the tertiary response level in control animals (see Table 3). The IgG1 responses to DNP-CGG in treated animals were somewhat

Table 3. Anti-I-A treatment impairs IgG1 anti-DNP responses more severely than IgG2a and IgG2b anti-DNP responses

Treatment*	Day of DNP immunization†	Splenic B cells on day of immunization,‡ estimated %	IgG anti-DNP responses,§ number impaired/number tested		
			IgG1	IgG2b	IgG2a
Anti-I-A on days 0, 2, 7, and 9	1	24	8/10	5/5	7/10
	8	8	6/10	0/5	2/10
	70	39	9/10	0/10	0/5
None	1	39	1/10	0/5	0/10
	8	39	0/10	0/5	0/10
	70	39	0/10	0/5	1/10

*Treated animals received 4 mg of anti-I-A antibody (0.5 ml of 10-3.6 ascites) on days 0, 2, 7, and 9.
†DNP-KLH (100 μg) on alum on day 1 and 8 and 50 μg of DNP-CGG on alum on day 70.
‡Estimated from Fig. 3 on indicated immunization day.
§Mice were bled 1 week after the first immunization and 2 weeks after the second and third immunizations. Anti-DNP responses, measured with a solid-phase binding RIA (17), were scored as impaired if they were <2 SDs below the mean of the appropriate control (non-anti-I-A-injected) and anti-DNP responses.

Table 4. Impairment of secondary IgG anti-carrier responses by anti-I-A treatment*

Treatment	Immunization	IgG anti-KLH responses, number impaired/number tested		
		IgG1	IgG2b	IgG2a
Anti-I-A	DNP-KLH twice	10/10	4/5	5/10
None	DNP-KLH twice	0/10	0/5	1/10

*Treatment, immunization, and bleed schedules, as well as responses and scoring are described in the legend for Table 3.

lower in magnitude and higher in affinity than were the majority of normal primary responses to DNP-CGG (data not shown). Thus, the impairment might be explained either by the belated recovery of B cells capable of giving rise to primary IgG1 anti-DNP responses or by the treatment-related induction of an active suppression that persists for IgG1 anti-DNP responses.

In either case these immunization studies demonstrate that the anti-I-A treatment and immunization protocol used in the earlier studies with (H,G)-A–L (3) selectively impair isotype responses to priming antigens encountered shortly after anti-I-A treatment has begun. The impairment of the IgG1 anti-DNP response when DNP-KLH is used as the priming antigen, is detectable considerably beyond the time of treatment. In the absence of data defining the biological roles of individual isotype responses, we suggest that these findings of selective isotype impairment be kept in mind when developing treatment protocols for human anti-Ia therapy lest individuals be accidently rendered nonresponsive for important isotype responses to environmental antigens encountered during therapy.

In essence, the findings we have presented suggest two potential mechanisms for explaining the effectiveness of anti-I-A injection in the immunosuppression and disease attenuation studies (3–5). First, the high serum anti-I-A levels generated by repeated anti-I-A injections may interfere with *I-A*-dependent antigen presentation or other regulatory interactions necessary for antibody production. Secondly, the rapid disappearance of B cells may deplete "virgin" precursors capable of giving rise to IgG antibody-forming cells and memory B cells needed for secondary responses. Both of these mechanisms are consistent with the available response data and both, in fact, probably exert important influences on antibody production in the anti-I-A-treated animal.

Our previous studies suggest that these influences may be mediated by an epitope-specific system (18) that independently regulates isotype antibody responses to individual epitopes on antigenic molecules. Studies with this system indicate that antigenic priming while B cell development or function is impaired results in the induction of a specific, persistent, T cell-mediated suppression of antibody production (18). However, the suppression generated in these "carrier/hapten-carrier" immunization studies tends to affect IgG2a and IgG2b antibody responses more than IgG1 responses (18) and, thus, differs from the impairment found in anti-I-A-treated animals.

The studies we have presented indicate that caution is needed before using the promising anti-Ia therapy for human autoimmune disease. In summary we have shown that (*i*) B cells are severely depleted by anti-I-A treatment; (*ii*) recovery of the B cells in treated animals lags considerably behind the disappearance of the injected antibody; (*iii*) genetic differences significantly influence sensitivity to B-cell depletion and recovery after anti-I-A treatment; (*iv*) introducing antigens while B cells are depleted and while I-A antigen on cells is potentially blocked by circulating anti-I-A markedly interferes with all immediate and some long-term responses to the antigens; and (*v*) some isotype components of antibody responses are more affected than others. Thus, combining these points with the general caution expressed previously (6), we suggest that anti-Ia therapy in humans should not be considered exempt from possible untoward consequences.

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