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Immunology

Soluble antigen abrogates the appearance of anti-protein IgG1-forming cell precursors during primary immunization

(B cells/immunologic tolerance/isotype switch/affinity maturation)

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ABSTRACT **The anti-human serum albumin (HSA) B-cell repertoire of C57BL/6 mice was examined by culturing splenocytes at limiting dilution following polyclonal stimulation with *Escherichia coli* lipopolysaccharide and a lymphokine mixture. The frequency of anti-HSA precursors was determined before and after immunization with alum-precipitated HSA and 10^9 killed *Bordetella pertussis* organisms, by submitting clonal supernatants to an ELISA. Anti-HSA IgG1-forming precursors were rare in unimmunized spleens, representing ≈1 in 500,000 splenocytes or only ≈100 cells per spleen. Between day 5 and day 7 after immunization, this figure increased to ≈20,000 cells per spleen. Over the following 3 weeks, there was a progressive increase in the mean optical density generated in the clonal ELISA, presumably due to affinity maturation of the B-cell population. When freshly deaggregated HSA was injected before or even up to 4 days after challenge immunization, the appearance of anti-HSA IgG1-forming cell precursors was largely prevented. The effect was most marked with 5 mg or 1 mg of soluble HSA, but impressive partial effects could be seen with as little as 10 μg of HSA if administered before challenge immunization. Most of the few clones seen after the higher doses of the toleragen appeared to make antibody of low affinity. The capacity to influence the B-cell pool by soluble antigen administered just 1–2 days before the sudden appearance of IgG1 precursors argues against the totality of the effect being due to T-cell-mediated suppression and in favor of a direct effect on B cells.**

Previous work from this laboratory (1–3) has shown that the preimmune B-cell repertoire of murine spleen contains surprisingly few cells with sufficient affinity for protein antigens (1, 2) or syngeneic intracellular structures (3) to bind in an ELISA when IgG1 antibody is the clonal product. Five days after challenge immunization with protein antigens and adjuvants, there is a sudden burst of appearance of IgG1 antibody-forming cell precursors (AFCP), most of which had already undergone an isotype switch *in vivo* (2) and were surface IgM-negative IgG1-positive cells. The relationship between these newly appearing B cells and the anti-protein IgM AFCP noted in unimmunized mice is not clear, but the former are unlikely, on kinetic grounds, to be the direct, unmutated progeny of the latter. Linton *et al.* (4) have put forward the challenging notion that the precursors of secondary B cells represent a separate lineage, susceptible to tolerance induction after immunization. The IgG1 AFCP could represent such cells. MacLennan and Gray (5) have argued that B cells are only recruited into the long-lived recirculating B-cell pool after a suitable encounter with antigen. Again, the newly recruited IgG1 AFCP could form part of this group of cells. The purpose of the present experiments was to test whether, and to what degree, soluble antigen could interfere with the upsurge of IgG1 AFCP after immunization. The results have provided an adult tolerance model consistent with direct negative signaling of recently activated B cells, but not negating the possibility that T-cell suppression is responsible.

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MATERIALS AND METHODS

Animals, Antigens, and Immunization. Specific pathogen-free C57BL/6 WEHI mice of both sexes and 8–10 weeks old at first immunization were used. Challenge immunization consisted of 100 μg of alum-precipitated human serum albumin (HSA) (Sigma) injected intraperitoneally (i.p.) together with 10^9 killed *Bordetella pertussis* organisms (Commonwealth Serum Laboratories, Melbourne, Australia). Some mice received a single i.p. dose of soluble HSA from 6 days before to 6 days after challenge immunization. This material had been freshly deaggregated by centrifugation at 15,000 × *g* for 10 min in a Heraeus Christ Biofuge A centrifuge. Some control mice received similarly treated ovalbumin (Sigma). Some groups of mice were serially bled from the orbital venous plexus, and serum samples were prepared for antibody assay.

Cell Preparation and Culture. Five thousand 3T3 BALB/c fibroblasts (Commonwealth Serum Laboratories, Melbourne, Australia) were aliquoted into flat bottomed 96-well microtiter trays in 100 μl. Culture medium (CM) was RPMI 1640 containing 100 μM 2-mercaptoethanol and 5% (vol/vol) fetal calf serum (Flow Laboratories). Spleen cell suspensions were prepared as described (6, 7) including 0.83% NH_4Cl for erythrocyte lysis followed by damaged and dead cell removal. Splenocytes were dispensed in 100 μl of CM containing *Escherichia coli* 0111:B4 lipopolysaccharide (Difco) at a final concentration of 20 μg/ml and a 10% (vol/vol) interleukin 4-containing CM from concanavalin A stimulated EL_4 thymoma cells (EL_4-BGDF) (8), making a final vol of 200 μl per culture. To determine IgG1 anti-HSA precursor frequency, cultures received 5000 splenocytes per well. A total of 288 replicate cultures was studied. Furthermore, to determine B-cell cloning efficiency, 48 replicate cultures were prepared at low input cell numbers (1.25–10 splenocytes per well). Two spleens were pooled for each time point. Cultures were incubated at 37°C in a humidified atmosphere of 10% CO_2/90% air for 7 days.

Antibody Assays of Serum Samples and Clonal Supernatants. An ELISA was used to detect and quantitate antibody formation in culture supernatants of the IgM and IgG1 isotype (1). U-bottomed, 96-well polyvinyl chloride plates (Costar) were coated overnight with 50 μl of affinity-purified sheep anti-mouse immunoglobulin (Silenus Laboratories, Hawthorn, Victoria, Australia) at 2 μg/ml in 0.2 M sodium carbonate buffer. For antigen-specific assays, a HSA solution (10 μg/ml) in mouse tonicity phosphate-buffered saline (pH 7.2) (MT-PBS) was used as the plate coat. An appropriate dilution of culture supernatant from each well was trans-

Abbreviations: AFCP, antibody-forming cell precursor; HSA, human serum albumin.

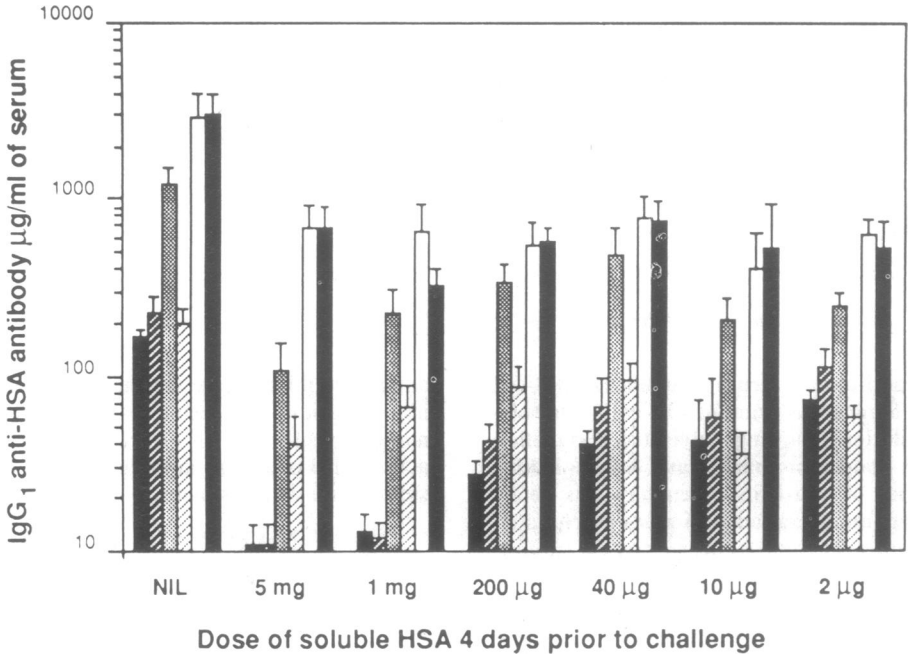

FIG. 1. Mean serum IgG1 anti-HSA antibody titers ± SEM at various times after immunogenic challenge in mice preinjected with soluble HSA 4 days prior to challenge. Each block of six vertical bars gives values from six successive bleeds, representing, from left to right, 10, 14, 28, and 56 days after primary immunization and 7 and 14 days after secondary immunization.

ferred into the precoated plates and held overnight at room temperature (RT). The diluent for the assay was MT-PBS containing 0.05% Tween 20, 1% newborn bovine serum, and 0.1% skim milk powder. After washing, the horseradish peroxidase-coupled anti-murine immunoglobulin with specificity for either IgM or IgG1 isotypes (Southern Biotechnology Associates, Birmingham, AL) was added for 4 hr at RT. The plates were washed before the addition of the substrate, 2,2-azinobis(3-ethylbenzthiazoline sulfonic acid) at 0.55 mg/ml in 0.1 M citric acid with 0.1% H_2O_2 (1 hr at RT). The absorbance of the wells was measured at 414 nm with reference wavelength at 492 nm, using a Titertek Multiskan MCC/340 (Flow Laboratories).

Anti-HSA antibody serum titers were measured by using duplicate serial 1:2 dilutions of serum starting at 1:100 and 1:1000 for the isotypes IgM and IgG1, respectively.

Statistical Analysis of ELISA Results. Computerized analysis of the ELISA was performed with software developed by A. P. Kyne (The Walter and Eliza Hall Institute). Serial 1:2 dilutions of the myeloma proteins MOPC 104E (IgM) and MOPC 21 (IgG1) (Bionetics Research Institute) on each test plate were used to standardize the assays. Hyperimmune anti-HSA mouse antiserum was used for the antigen-specific ELISA. A cubic spline curve-filling technique was used to fit a curve through all points of the standard curves, and the mean clone antibody content for each group and antibody content of individual wells were computed from this.

Poisson analysis (9) was used to determine the frequency

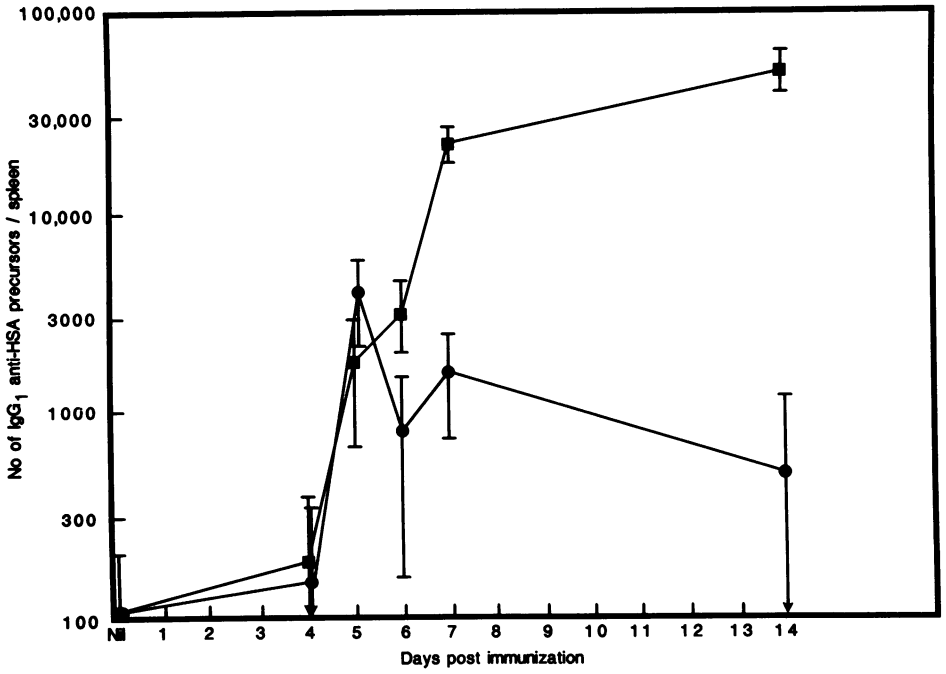

FIG. 2. Number ± 95% confidence limits of clonable IgG1 anti-HSA AFCP/spleen at various times after immunization in control mice receiving no preinjection or 5 mg of ovalbumin 4 days before immunogenic challenge (■) or tolerant mice receiving soluble HSA before challenge (●); 5 mg for the 4-, 5-, 6-, and 7-day points and 1 mg for the 14-day point.

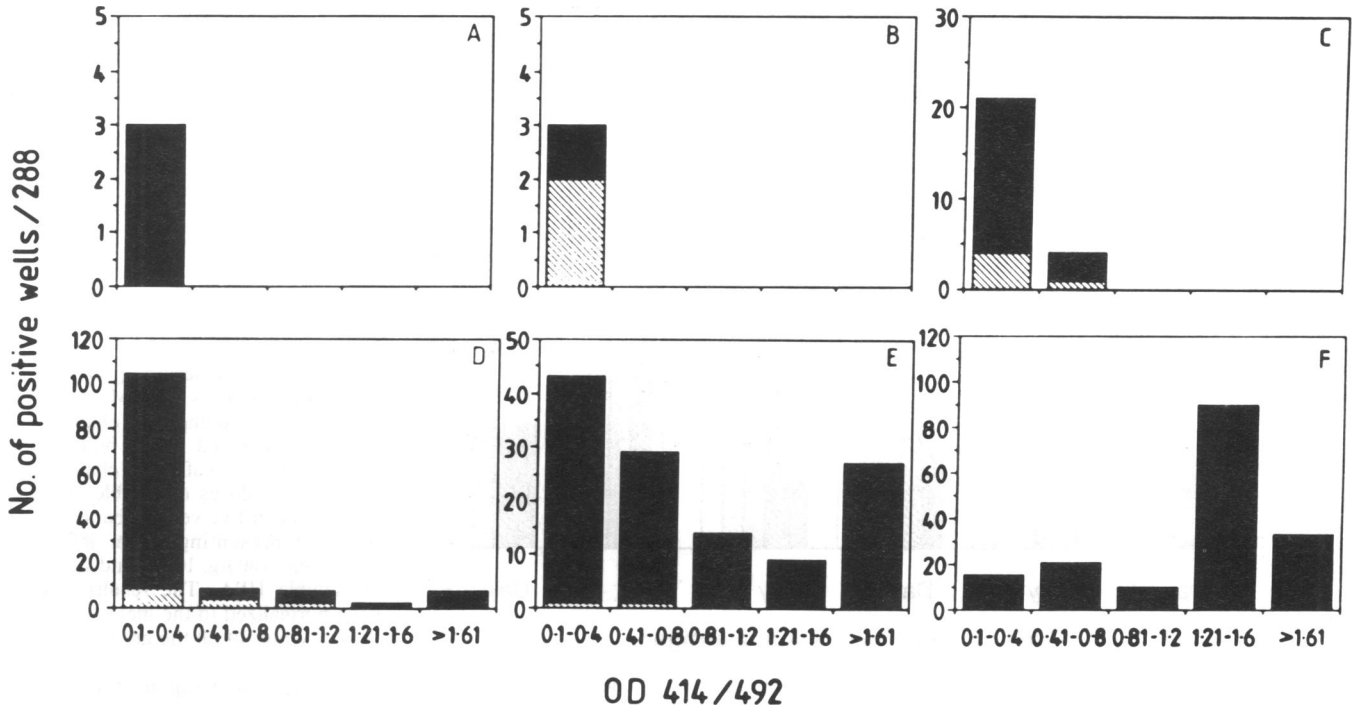

FIG. 3. ELISA OD values for supernatants of limit dilution culture wells, representing IgG1 anti-HSA antibody. Solid areas, control values; shaded areas, values from tolerant mice receiving preinjected HSA. (*A*) Unimmunized mice. (*B–E*) Immunized control and tolerant mice killed 4, 6, 7, and 14 days after challenge. (*F*) Immunized control mice killed 28 days after challenge. Experiment *F* had no tolerant companion group.

of immunoglobulin-secreting precursors and the maximum likelihood estimator for linear regression analysis (10).

RESULTS

Effects of Soluble HSA 4 Days Prior to Challenge on Serum Anti-HSA IgG1 Titers. Groups of eight mice were injected with a single i.p. dose of soluble HSA 4 days prior to challenge immunization and were bled serially. Eleven weeks after the first immunization, they were given booster injections of 100 μg of alum-precipitated HSA without pertussis and were bled 7 and 14 days later. The results are given in Fig. 1. Control mice first showed IgG1 antibody above background ≈7 days after immunization and made a good primary response, peaking 4 weeks after challenge. They also gave a rapid secondary response increasing to 3 mg of antibody per ml. Mice preinjected with 5 mg or 1 mg of soluble HSA made a 10- to 20-fold lower primary response, the reduction at 10 and 14 days being somewhat more profound than that for later bleeds. Lower toleragen doses yielded lesser effects, with a rather flat dose–response relationship. The effects on the secondary response were also substantial, with a 3- to 4-fold reduction in antibody titer but a remarkable retention of this effect even at the lowest dose of soluble HSA.

Anti-HSA IgG1 Precursors Following Challenge Immunization. Preliminary experimentation established that, as with keyhole limpet hemocyanin as an immunogen (1), the proportion of apparent anti-HSA IgM AFCP did not increase significantly after immunization. Many wells would have scored positive even without coating ELISA trays with HSA, representing "anti-plastic" clonotypes (11). Accordingly, the chief emphasis of this study was on IgG1-forming clones. Further work showed that mice preinjected with 5 mg of keyhole limpet hemocyanin or ovalbumin yielded anti-HSA IgG1 AFCP in numbers not significantly different from those of mice left uninjected prior to challenge immunization, so these three different types of controls were pooled. Fig. 2 represents a typical experiment in which the toleragen was 5 mg of soluble HSA, except for the 14-day group, in which 1 mg was used. In each case, soluble antigen was administered 4 days before challenge. The results show no significant increase in the small number of AFCP 4 days after immunization, and then an approximately exponential increase. Soluble antigen preinjection had no effect on the few precursors noted on day 4 and did not prevent the first abrupt increase on day 5 but entirely abrogated the later increases. The 10- to 20-fold lowering achieved paralleled the similar lowering in primary response serum IgG1 antibody levels.

Affinity Maturation of Antibody Formation. Fig. 3 analyzes the OD distribution of positive wells as a function of time after immunization. Note the scale variation in the vertical axes to accommodate the much larger number of positives on

Table 1. Mean clone anti-HSA IgG1 antibody found in ELISA as a function of time after immunization

Time after primary immunization, days	Mean antibody bound per clone, pg
0	184
4	168
5	248
6	256
7	360
14	1040
28	2000

Mice were challenged with a single dose of 100 μg of alum-precipitated HSA and pertussis. Three replicate trays (288 cultures) of 5000 splenocytes per well were analyzed for IgG1 anti-HSA bound in ELISA after 7 days in culture. Antibody values were adjusted for clonal overlap (9). The numbers of clones from tolerant mice were too small to give reliable values but in general showed less antibody bound than in the controls. The mean antibody bound for the 14- and 28-day cultures understates the true value as a significant number showed OD at the maximum read by the Multiskan (Flow Laboratories) instrument and would have required dilution for accurate analysis.

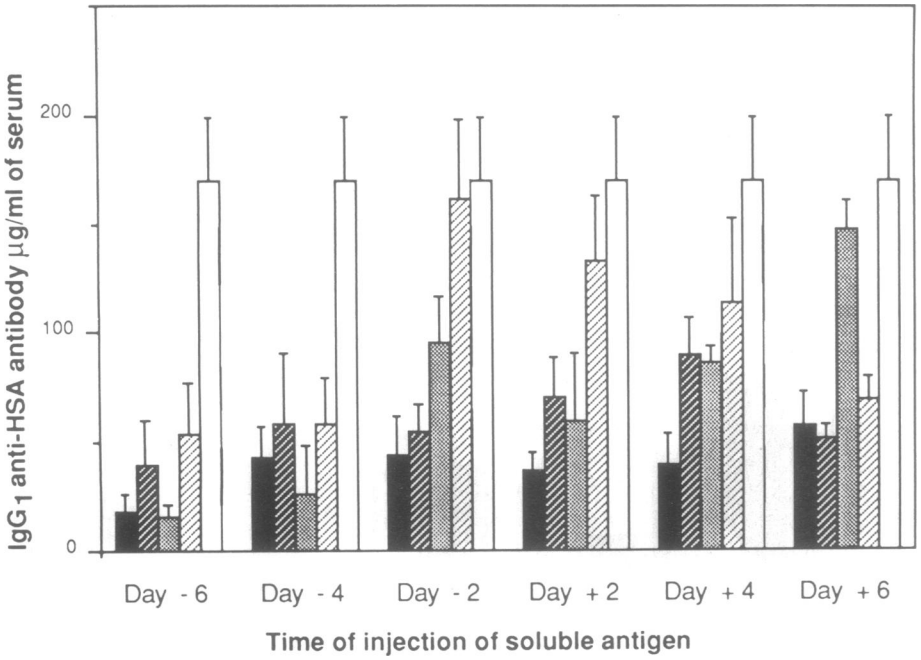

FIG. 4. Mean serum IgG1 anti-HSA antibody titers ± SEM 14 days after primary immunogenic challenge from mice injected at the indicated times before (−) or after (+) challenge with various doses of soluble HSA. Each block of five vertical bars gives results representing, from left to right, 1 mg, 100 μg, 10 μg, and 1 μg or no soluble HSA. The results have been normalized to the mean control antibody titer of all the groups.

later days. Note further that results have not been adjusted for clonal overlap. All of the few clones found without immunization or at 4 days exhibit a low OD, and as time progresses, clones with higher ODs increase and come to dominate the population by 28 days. The few clones noted in tolerant mice were chiefly of low OD but their number was so small that more work would be required to assert definitively that the OD spectrum was different from that of controls. ELISA with anti-murine immunoglobulin as the capture layer (12) shows that IgG1-forming clones in this system can produce a mean of 17.6 ng of IgG1 after 8–9 days of culture and ≈2-fold less after 7 days of culture, as in these experiments. An OD of 0.4 represents ≈600 pg of bound antibody in Fig. 3, so clearly only a minority of the IgG1 formed bound to HSA under the conditions of the ELISA. This indicates the low affinity of the few preimmune IgG1 AFCP. Table 1 gives results adjusted for clonal overlap. It shows the increased binding per clone with increasing time after immunization.

Effects of Time of Injection of Soluble Antigen on Tolerance Induction. Figs. 4 and 5 give the results of an extensive series of experiments in which both the dose of toleragen and the timing of its injection were varied widely. Because these experiments spanned several months, with inevitable variation in control values, they have been normalized. Fig. 4 shows serum titers normalized to different control values and Fig. 5 shows AFCP numbers normalized to various cloning efficiencies on different days. Fig. 4 shows that low doses of soluble HSA are most effective in lowering 14-day serum antibody titers if given at least 4 days before immunogenic challenge, whereas higher doses can work even if delayed until after challenge. However, neutralization of serum antibody by residual soluble antigen must be considered a possibility in the latter case. Fig. 5 shows the effects of toleragen on the number of anti-HSA IgG1 AFCP found in the spleen 14 days after challenge. The highest dose used (1 mg) virtually abrogated the appearance of AFCP at any time of administration up to 4 days after challenge, but significant

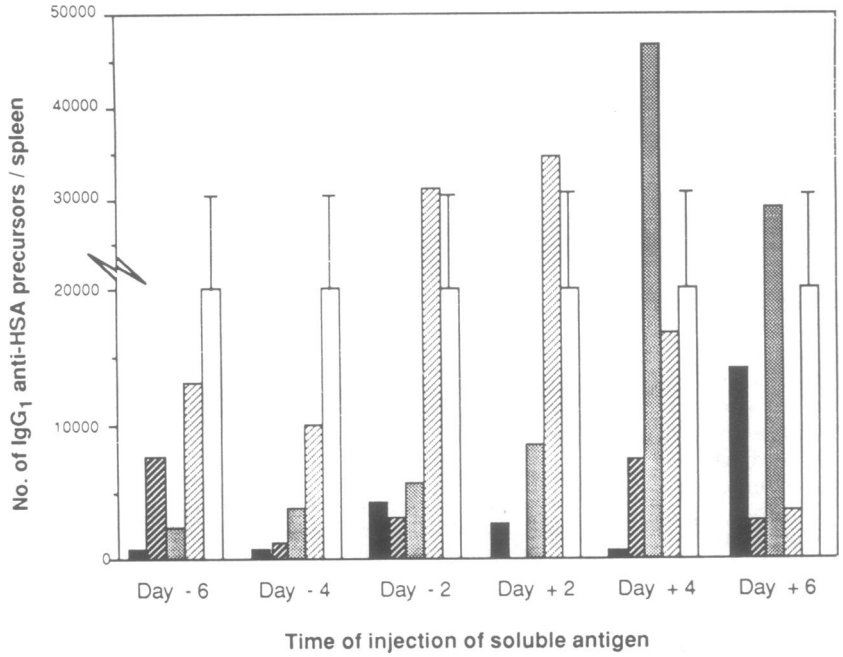

FIG. 5. Number of clonable IgG1 anti-HSA AFCP/spleen 14 days after primary immunization in mice injected at the indicated times with various doses of soluble HSA. Results are presented as in Fig. 4. The results have been normalized to the mean IgG1 cloning efficiency of all experiments in the series.

escape occurred if the injection was delayed to +6 days. The dose–response profile for day +6 is difficult to explain, especially the 1-μg result given the inefficacy of this dose of toleragen on all other days of administration, but in general the results suggest that the later the toleragen is administered, the higher the dose required. Mean clone antibody content for controls was consistent with data from Table 1. Partially tolerant animals in general showed a lower mean antibody content than controls (data not shown).

DISCUSSION

This paper probes the B-cell repertoire of the mouse through utilizing the capacity of interleukin 4 to cause polyclonally stimulated B cells to switch to extensive IgG1 antibody formation (13). The paucity of anti-HSA IgG1 AFCP in unimmunized spleen is of interest as it shows that the large virgin repertoire contains few members capable of forming antibody of good affinity to a typical foreign protein. Of equal interest is the fact that these few precursors do not seem to divide over the first 4 days after immunization, as their number in the spleen does not increase significantly. At 5 days, a marked increase is apparent. This could represent a combination of division and recruitment of new cells from the bone marrow and elsewhere into the spleen. It could also mean that a different population of B cells, not the progeny of those active at 4 days, has now passed a stage in ontogeny where the cells become inducible into clonal expansion and antibody production under our *in vitro* conditions (4). The first burst of IgG1 AFCP seems unlikely to be the result of immunoglobulin *V* gene hypermutation (14, 15). First, the antibody affinity does not appear to be higher (Fig. 3); second, studies on T-cell-dependent anti-hapten responses suggest that mutations are uncommon before 10–12 days after immunization (16, 17). This question requires direct study. The later affinity maturation at days 14 and 28 is likely to be due to *V* gene mutation.

The tolerance induced by soluble antigen could be explained in several ways. While the exact nature of T-cell suppression remains to be elucidated, this could be a cause, particularly as the *in vivo* isotype switch is dependent on T-cell help. A counterbalancing T-cell suppression could impede the appearance of IgM-negative IgG-positive anti-HSA AFCP. Most studies of T-cell suppression (e.g., see refs. 18–20) depend on soluble antigen given at least 1 week before challenge immunization. Thus, the finding that soluble antigen can be delayed until just before the first increase of IgG1 AFCP argues somewhat against T-cell suppression being the only mechanism at work. Several types of direct effects of soluble antigen on B cells could be envisaged. First, as Figs. 1 and 4 show, tolerance is not complete in this model. Some antibody is made that could complex with soluble antigen. Such complexes, particularly in the zone of slight antigen excess, are profoundly suppressive of B-cell activation, negative signaling being delivered via the B cell's Fc receptor (21–23). Second, large doses of soluble antigen could interfere with the interaction between B cells, antigen-presenting cells, and helper T cells, which is required for isotype switching and memory cell generation (5). Third, the mechanism that Linton *et al.* (4) have postulated may be at work. They consider that B cells emerge from the bone marrow as two distinct populations, one designed to produce a primary antibody response and the other to react to antigen not by antibody production but by memory cell generation. They postulate that the latter population of secondary B cells passes through a stage of tolerance susceptibility shortly after activation; the rationale for this is to provide a mechanism whereby B cells fortuitously mutating toward anti-self reactivity could be censored. Of course, these various mechanisms are not mutually exclusive. The possibility exists that the effects of small doses of soluble antigen given well before challenge are due to T-cell suppression, whereas those of large doses of antigen given after challenge may be due to direct effects of antigen or immune complexes on recently activated B cells. Adoptive transfer studies are required to distinguish between these alternatives.

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