IMMUNOGLOBULIN SYNTHESIS IN MICE

SUPPRESSION BY ANTI-ALLOTYPE ANTIBODY*

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Differentiation of the immune system poses the following novel problems in control of expression of genetic material. For all autosomal loci, expression of a locus in a given cell always means expression of both alleles at the locus; however, immunoglobulin loci although autosomal, do not conform to this rule (1). Mature plasma (antibody-producing) cells produce immunoglobulins specified by only one or the other of the two alleles at the immunoglobulin locus being expressed by the cells; e.g. in mice, for a cell making γG_{2a}, the cell produces either Ig-1a or Ig-1b (2).

Although much is known about X-chromosome inactivation (the "Lyon effect") (3), there is little to suggest how and when a single autosome, or part of an autosome, may be selectively inactivated in a cell. Studies presented in this paper on the suppression of production of allotype in young animals exposed to antibody reacting with the allotype have yielded results bearing on this question of the committment of a cell to production of a single allotype.

Following the demonstration by Dray (4) that severe prolonged suppression of the synthesis of paternal gamma globulin isoantigen (allotype) resulted from the gestation of a heterozygous rabbit in a mother immune to the paternal allotype, the attention of several laboratories focused on the effects on mother and/or fetus of immunoglobulin incompatibilities. Mage and Dray (5) have shown that the depression of synthesis after exposure to anti-paternal allotype antibody is evident for up to 3 yr. In the same publication they showed that similar depression could be obtained by injection of anti-paternal allotype at birth. Dubiski and Fradette (6) confirm this latter result in a more recent publication.

Attempting to extend this work to the mouse, Lieberman and Dray (7) were unable to demonstrate a similar suppression of paternal allotype in response to maternal anti-paternal antibody. The majority of their offspring from immune mothers died either at birth or just prior to birth under conditions where the mother appeared unable to deliver the litter normally. Of the survivors, all appeared to have normal levels of paternal allotype.

In contrast with Lieberman and Dray, we have been able to demonstrate, in the hybrid mouse, suppression of paternal allotype production in response to maternal

anti-paternal allotype. We find the period during which allotype is absent or below detectable limits is short, measurable in weeks rather than months, but that the period during which the allotype levels remain below the controls extends considerably beyond this time (8). Extension of these studies on the suppression in heterozygotes is presented in this publication.

By taking advantage of the fact that the mouse continues to pass antibody to the young throughout nursing, we have been able to show also (a) that foster nursing of inbred (homozygous) animals on mothers immune to the allotype of the foster-nursed homozygotes causes suppression of production of allotype by these homozygous progeny and (b) that injection of small amounts of anti-allotype antibody into homozygous animals of the allotype against which the antibody is directed suppressed production of the allotype. That is, we have been able to create an experimental dysgammaglobulinemia where synthesis of one class of immunoglobulins, determined at the Ig-1 locus, is suppressed.

Materials and Methods

Mice.—The strains of mice used for this work were C57BL/10Hz and BALB/cCrgl/Ga.

Maintenance of Mice.—Each breeding cage contained three females and one male per cage. Pregnant animals were removed and allowed to deliver separately.

Progeny to be foster-nursed were obtained by allowing the mothers to deliver on a platform made of a wire screen with approximately ½ inch mesh. Offspring dropped through the holes onto a bed of shavings. They were recovered as soon as possible, transferred to a 37°C incubator for approximately 10 min and then transferred to the foster mother, whose young had been removed shortly before. We have observed no nursing of offspring by the natural mother with this procedure.

Progeny were weaned 21–28 days after birth, separated into two cages by sex and maintained this way thereafter. Nonlittermates were never mixed. Serum samples were taken from progeny at weekly intervals by incision of the tail artery. Sera were prepared and stored as previously described (9).

Antisera.—Antisera were prepared as previously described (9).

Maternal Immunization.—BALB/C females, age 6–10 wk were immunized as previously described, i.e., with a primary subcutaneous injection of 10 µl of C57BL/6 anti-H 2d serum in complete Freund's adjuvant followed 30 and 37 days later by 20 µl intraperitoneal injections of the same serum (9). Additional "booster" injections were given at approximately monthly intervals, between litters. Virtually all females immunized produced precipitins demonstrable by agar gel diffusion. Those which did not were discarded.

Serological Techniques.—Screening for progeny Ig-1b immunoglobulin was done by agar gel diffusion. A serum scored as positive by this method contained at least 0.03 mg/ml of Ig-1b.

Quantitation of Ig-1b.—Ig-1b in serum samples was measured by inhibition of precipitation of ^{125}I-labeled Ig-1b by an alloantiserum prepared in Ig-1a animals. This method has been previously described (9).

Statistical Analysis.—

(a) *Comparison of age of expression of Ig-1b between progeny from immune and nonimmune mothers:* For each litter, the first week at which 50% or more of the animals in that litter demonstrated circulating Ig-1b was chosen as the median response time. The null hypothesis is that litters from immune and nonimmune mothers do not differ with respect to their median response times. The test is based on the number of times the score for a litter from an immune

mother exceeds that for a litter from a nonimmune mother minus the number of times the score for a litter from a nonimmune mother exceeds that for a litter from an immune mother. A large positive value of this quantity suggests rejection of the null hypothesis in favor of the alternative that the litters from immune mothers have on the average later median response times. In cases where the exact score for a litter was unknown we conservatively assigned the earliest possible median response time to the litters from immune mothers and the latest possible median response time to litters of nonimmune mothers. This was done to assure that the significance of the result is underestimated. This procedure is a variation of the Mann-Whitney rank test (10).

(b) *Variation of response times of litters within the two "treatment" groups: The null hypothesis:* the distributions of response times are identical from litter to litter within treatment groups, observed differences being due solely to sampling variation; the alternative hypothesis: these distributions vary systematically from litter to litter, even within the same treatment group.

Analysis was somewhat complicated by the fact that the information available varied with litter—some litters were not tested before day 42 and hence could not be directly compared with those tested on day 25 as there was more information available on the latter which we wanted to use in our test. Thus, the litters were divided up into subgroups within group according to the amount of information available, and the analysis carried out independently for each of these subgroups.

The Kruskal-Wallis rank test of homogeneity was used (11). For each table a conservative χ^2 value was computed (conservative because ties were ignored). All but two of these χ^2 were significant at the 0.05 level, and those two were for the subgroups in which the least information was available. All χ^2 were then pooled for an over-all test of homogeneity.

RESULTS

The data in Table I show that animals either heterozygous or homozygous for Ig-1b exposed to mothers or nurses immune to Ig-1b are slower than controls to reach the threshold of detection for Ig-1b (0.03 mg/ml) and have less Ig-1b than controls when tested several weeks after synthesis has begun.

There is a delay (approximately 25 days) in expression of Ig-1b in heterozygotes born to immune vs. nonimmune mothers, and the depression of Ig-1b level at 9 wk to 20% of control level; a delay (also approximately 25 days) in expression of Ig-1b is found between homozygotes nursed on immune vs. nonimmune mothers and the depression of Ig-1b level at 8 wk, of the suppressed animals, to 30% of the control. Finally, there is a delay (approximately 13 days) in expression of Ig-1b in homozygous animals nursed on nonimmune mothers but injected at 7 and 14 days of age with anti-Ig-1b, and the depression of the Ig-1b level in these animals at 8 wk to 70% of control level. The time at which all animals had at least 0.03 mg/ml Ig-1b was used for quantitative comparison of Ig-1b levels done by inhibition of precipitation of ^{125}I-labeled Ig-1b.

In Fig. 1, the cumulative percentages of animals positive for Ig-1b are presented as a function of age for the various groups.

In Fig. 2, the appearance of Ig-1b positive litters is presented as a function of litter age. These curves are based on the same data as Fig. 1. A litter is scored as positive at the time when 50% or more of the animals comprising it are positive. Litters generally take from 1 to 3 wk to become 100% positive.

Control litters (born to nonimmune mothers) are distributed roughly normally with a mean around 40 days, whereas litters born to immune mothers peak around 60 days. A nonparametric test for the significance of the difference in distribution of the two populations of litters (Mann-Whitney U test) gave a normal score value of 3.8, which indicates a one-sided significance level of p < 0.0001.

By comparing the distributions of responses of individual animals within various litters within the two subgroups (progeny from immune and nonimmune

TABLE I

Suppression of Ig-1b in Progeny due to Maternal Anti-Ig-1b

Progeny	Genotype	Foster mother	Treatment of offspring	Number of litters	Number of animals	Age at which half the animals demon-strate Ig-1b	Serum antigen level relating to control	
							Level	Day level done
Hetero-zygotes	Ig-1^b/Ig-1^a	Immune to Ig-1b	...	16	84	56	0.2 (3)	72
	Ig-1^b/Ig-1^a	Nonimmune Mother	...	18	105	33	1.0 (3)	72
Homo-zygotes	Ig-1^b/Ig-1^b	Immune to Ig-1b	...	3	16	51	0.3 (4)	56
	Ig-1^b/Ig-1^b	Nonimmune	Injected with anti-Ig-1b	3	13	34	0.7 (3)	56
	Ig-1^b/Ig-1^b	Nonimmune	...	4	24	24	1.0 (3)	56

Heterozygotes are of the cross BALB/C female × C57BL/10 male or BALB/C female × (C57BL/6 × DBA/2) male. In the latter case only the Ig-1b progeny were used. Homozygotes are C57BL/10. Foster nurses are BALB/C. Homozygotes are separated at birth from their natural mothers before any opportunity to nurse occurs. Transfer to the foster nurse is made within 6 hr. Mothers and foster mothers were immunized prior to mating and then boosted periodically as previously described. Progeny injected with anti-Ig-1b were given 30 μl of a pooled antiserum at 7 and 14 days of age. The number of animals tested does not represent all animals in a litter, since in some cases animals were removed at random, at birth, for use in experimental studies. Agar gel double diffusion was used to screen for expression of Ig-1b. A positive response equals approximately 0.03 mg/ml of Ig-1b. The age at which half the animals demonstrate Ig-1b in each group was taken from the data in Fig.1. Serum levels of Ig-1b were estimated by inhibition of precipitation of ^{125}I-labeled antigen, then expressed relative to the control. The number of litters averaged appears in parentheses after the value.

mothers), we show that the response times vary systematically from litter to litter and cannot be accounted for by sampling variation from the entire treatment group. To assess the overall statistical significance we pooled χ^2 values from several independent Kruskal-Wallis rank tests for homogeneity done on groups for which comparable data was available, within the groups of progeny from immune and nonimmune mothers. The χ^2 value obtained was 100, with 25 degrees of freedom, which yielded a normal deviate of 10.7 (p < 0.0001).

The results of detailed quantitative studies of the level of Ig-1b attained as a function of age are presented in Fig. 3. The data from three litters were pooled for each curve. Consistent with the data presented earlier, Ig-1b appears later and Ig-1b levels rise more slowly in progeny of immune mothers than in con-
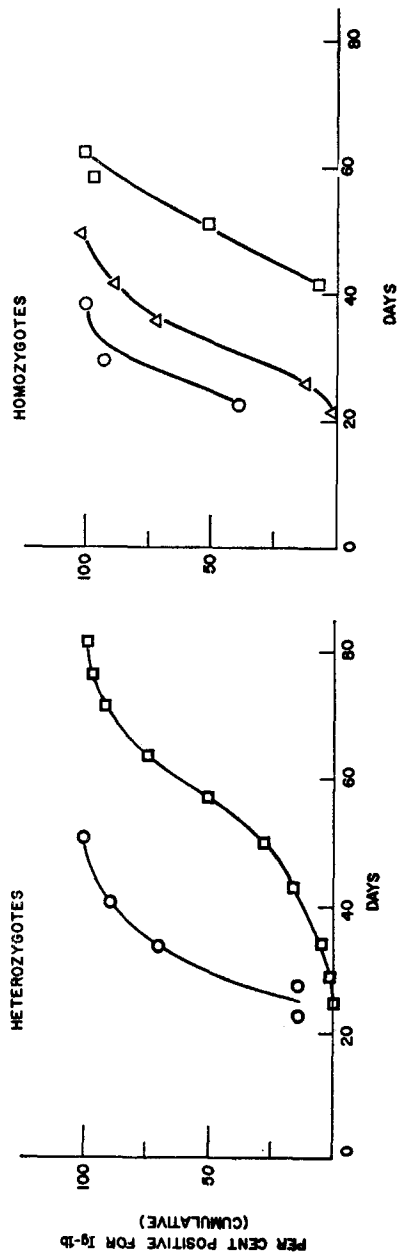
Fig. 1. Cumulative percentage of Ig-1b positives as a function of age. Heterozygotes: open circles, control progeny from nonimmune mothers; open squares, suppressed progeny from immune mothers. Homozygotes: open circles, progeny foster-nursed on nonimmune mothers; open triangles, progeny foster-nursed on nonimmune mothers injected with anti–Ig-1b at 7 and 14 days; open squares, progeny foster-nursed on immune mothers. See legend from Table I for details of experiment.

trols. Even at the end of the experiment, where the animals were 4 months of age or more, the progeny exposed to immune mothers have not achieved the same Ig-1b levels as controls. Curves for heterozygotes and homozygotes are given.

Previously (8), we presented arguments showing that only if the synthesis of Ig-1b was suppressed could the observed decrease in Ig-1b levels in progeny exposed to immune mothers be accounted for. That is, we concluded that
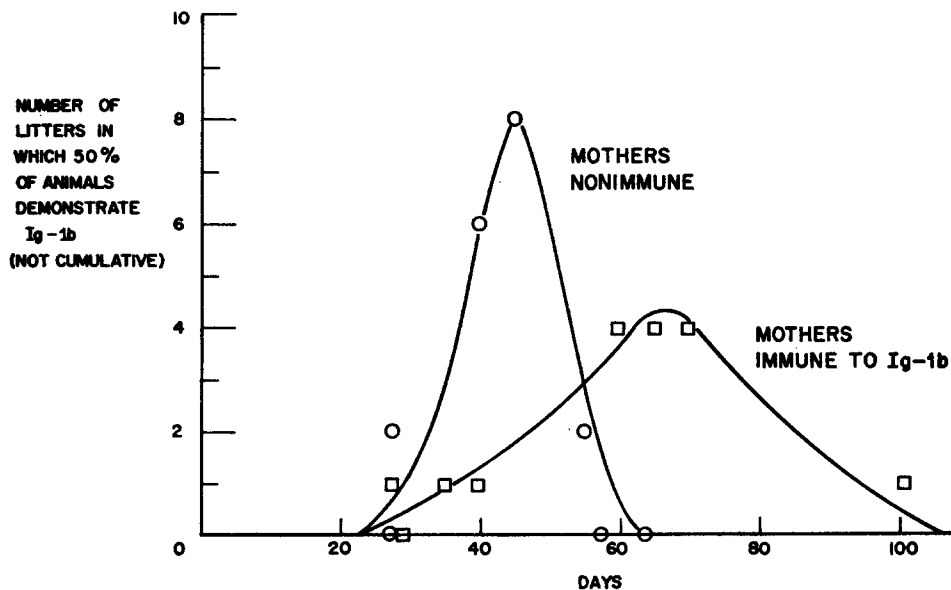

FIG. 2. Number of litters in which 50% of animals are positive as a function of age of litter. Data are not cumulative. See legend for Table I for details of experiment.

elimination of Ig-1b by maternal antibody is not solely responsible for the differences between experimental and control animals. Using the data presented in Fig. 3, we have confirmed this conclusion and extended it to give an estimate of the degree of suppression of synthesis in the heterozygote. This was done as follows:

The curves in Fig. 3 represent the amount of Ig-1b present per animal at the indicated age. For any given age this amount is equal to the amount of Ig-1b produced minus the amount lost due to catabolism, blood sampling, and, where applicable, absorption by maternal antibody. Using the curve for control animals, and making appropriate estimates for losses due to catabolism and sampling, the rate of Ig-1b synthesis in animals from 0 to 180 days of age was determined. Based on this rate, calculations were made predicting the amount
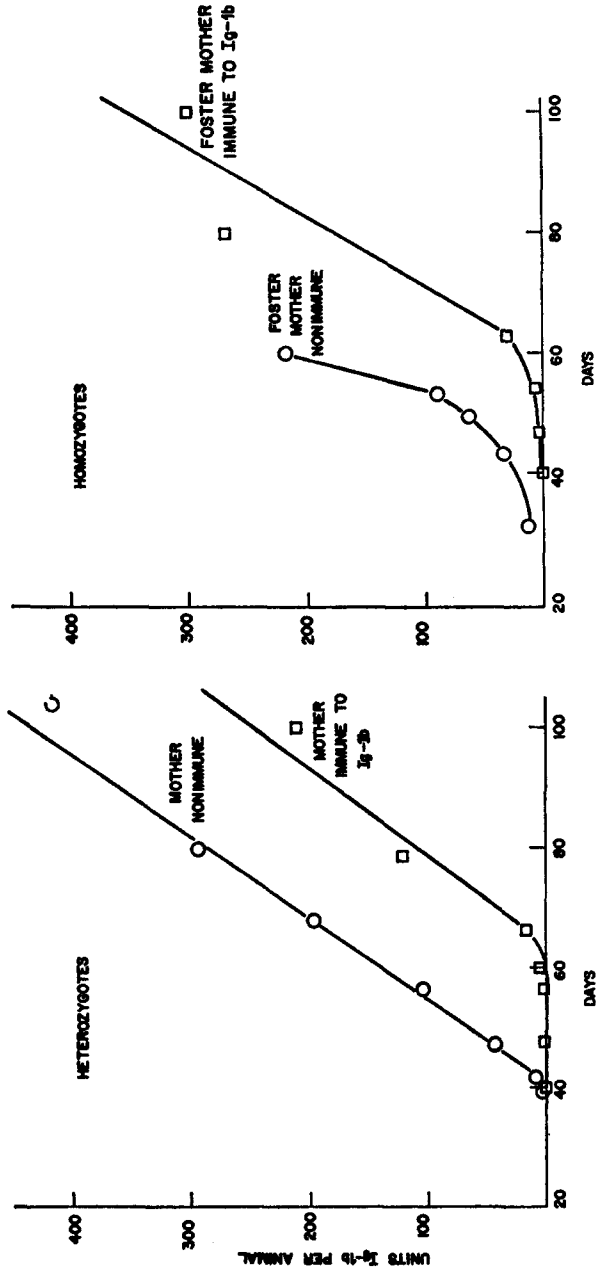
Fig. 3. Increase in Ig-1b with age of animal for suppressed and nonsuppressed homozygotes and heterozygotes. A unit of Ig-1b equals the amount of Ig-1b in 1 μl of a standard pool of C57BL/6 adult normal serum. Units per animal are calculated from the determined units per ml serum and the estimated gamma globulin space (8.8% body weight). Curves represent averages of three litters of approximately six animals per litter.
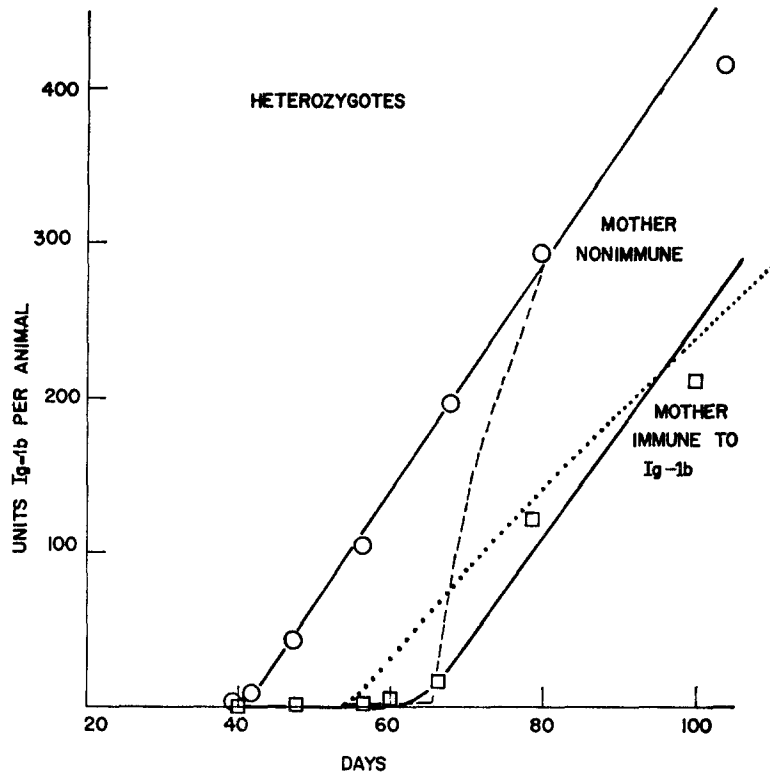
FIG. 4. Calculated increase in Ig-1b level in heterozygotes. Solid curves reproduced from Fig. 3. Dashed curve (---) was calculated on the basis of 500 units of Ig-1b removed but no change from the rate of production of Ig-1b found for the controls. Dotted curve (· · ·) was calculated on the basis of 150 units of Ig-1b removed and the rate of production of Ig-1b equal to one-half the control rate. A half-life of 6 days was used for both curves. Corrections for the amount of Ig-1b withdrawn in serum sampling were made. For $t = 0, 1, 2, \ldots 179$, the rate of production of Ig-1b at time t (PRO$_t$) was found by equation (1) and substituted in equation (2) to determine the amount of Ig-1b expected at time t (Calc Ig$_t$).

(1) $\mathrm{PRO}_{(t+1)} = (\mathrm{Ig}_{(t+1)} - \mathrm{Ig}_t) + \left(\dfrac{\ln 2}{T} + k \right) \mathrm{Ig}_t$ and

(2)   Calc $\mathrm{Ig}_{(t+1)} = $ Calc $\mathrm{Ig}_t + q \cdot \mathrm{PRO}_t - \left(\dfrac{\ln 2}{T} + k \right)$ Calc $\mathrm{Ig}_t - \vartheta$

where Ig$_t$ is the observed Ig-1b level in control at time t; T, the half-life in days (6, in curves in figure); k, the fraction of total Ig-1b present in the animal removed by sampling averaged per day (0.1/7 in curves in figure); q, the fraction by which the rate of production (PRO) is altered; and ϑ, the amount of Ig-1b withdrawn at $t = 0$.

of Ig-1b expected to be present in experimental animals after initial removal of several amounts of Ig-1b equivalent to what might be absorbed by maternal antibody. Calculations were also made predicting the effect of decreasing the rate of Ig-1b syntheses in combination with initial removal of several amounts of Ig-1b.

Two predicted curves are shown in Fig. 4 along with the observed curves reproduced from Fig. 3. Rates of synthesis were determined assuming constant half-lives of 4 days and 6 days and an average of 10% removal due to sampling each 7 days starting at 40 days. Calculations based on these rates showed that initial removal of 100 to 400 units of Ig-1b yields predicted curves which merge with and become indistinguishable from the control curves by 90 days at the latest. This is contrary to experimental observation as levels in the suppressed animals do not reach the levels of the controls even by 20 wk. The predicted curve for 400 units of Ig-1b removed and a half-life of 6 days is shown. The period during which there is no detectable Ig-1b is slightly longer but the rate of increase of Ig-1b, once it appears, is far greater in this curve than is observed in suppressed animals. The observed curve is reasonably closely approximated by a reduction of the rate of synthesis by one-half and the removal of 150 units of antigen (6-day half-life). The predicted curve for these conditions is also presented.

DISCUSSION

Allotype suppression by anti-allotype antibody in mice has now clearly been shown to be due to a suppression of synthesis of immunoglobulins carrying the allotype. Genotypically Ig-1b mice exposed to maternal anti-Ig-1b pre- and postnatally or just postnatally (via nursing) show a delay in acceleration of the rate of Ig-1b allotype synthesis compared to control mice. A slightly lower synthetic rate persists for 1 to 2 months in the absence of further antiallotype treatment. However, the extreme suppression of allotype synthesis found in rabbits by similar antibody treatment is not seen in mice. Also, the compensatory increase in the nonsuppressed allotype often observed in rabbits has not been detected in mice, although such compensatory synthesis in mice would be difficult to document because of the less severe suppressions so far obtained.

With mice, allotype suppression in homozygotes has been obtained both by nursing from birth with foster mothers immunized against the allotype or by injection of antiserum into mice foster-nursed on nonimmune mothers of another allotype. Similar experiments with homozygous rabbits have not been reported.

A hypothesis attempting to explain allotype suppression in both mice and rabbits must account for the difference in severity and duration of suppression in the two species. In contrast with the mouse, greater than 90% suppression

has persisted for the life of rabbits treated only neonatally with anti-allotype serum. The following hypothesis is put forward as consistent with the available data and as subject to experimental test in several ways. Concepts in the scheme have been freely drawn from many sources, (see, e.g., the review, reference 12).

For convenience in writing only, the following deals only with the H chain control. It is meant to refer equally to L chains. Let us suppose that the embryonic immunoglobulin stem cell is totipotent with respect to class and allotype of immunoglobulin its progeny can produce. It contains two chromosomes, both of which carry the loci for all classes of immunoglobulin, but each of which, in an allotypic heterozygote, carries a different set of alleles at the immunoglobulin loci. As differentiation towards immunoglobulin production progresses, one of the two immunoglobulin chromosomes (or immunoglobulin chromosome regions) of the embryonic stem cell, either paternal or maternal at random, becomes irreversibly inactivated. This might be much in the same fashion as the X-chromosome inactivation (3). Further differentiation then leads to the partial de-repression of one of the H chain immunoglobulin loci on the active chromosome, and the production of a few (perhaps only one) molecules of the immunoglobulin class specified at the de-repressed locus. At least one of these immunoglobulin molecules then remains associated with the cell, identifying the de-repressed locus and (directly or indirectly) repressing the other H chain loci. After some time, possibly after cell division, a different immunoglobulin H chain locus on the active chromosome may become de-repressed, bringing about repression of the old locus and replacement of the old immunoglobulins, thus recommitting the cell to production of a different class of immunoglobulin. This tentative committment will then hold until the next revolution.

We postulate that there is a self-replenishing stem cell population in the immunologically mature individual consisting entirely of cells which (a) have one H chain immunoglobulin chromosome active and the other irreversibly inactivated, (b) have one H chain locus on the active chromosome partially de-repressed, (c) have one or more molecules specified by the de-repressed locus associated with the cell, but (d) are only tentatively committed to the immunoglobulin they are producing and periodically shift to de-repression of another locus on the active chromosome and production of a different immunoglobulin.

The identifying immunoglobulin on the stem cell (perhaps situated in association with the cell membrane) is accessible to external reactants, such as antigens and anti-allotype antibody. However, whereas combination with antigen may lead to differentiation of the tentatively committed cell into the irreversibly committed, fully de-repressed plasmacytoid line (i.e., to immunization), combination of the identifying immunoglobulin with anti-allotype antibody results in the removal of the cell from the population able to give rise to the allotype, either through direct killing or through transformation into a different cell type

(lymphoblastoid transformations?). This removal may be analogous to the mechanism by which tolerance is produced by appropriate antigenic stimulation.

The difference in duration of suppression between mouse and rabbit are predicted by this model. In the mouse the allotype antigens with which the suppressing antiserum reacts are on one class of immunoglobulins, i.e., are determined at only one locus on the immunoglobulin chromosome, Ig-1b, which codes for γG_{2a} globulin. Therefore, only cells producing Ig-1b are attacked. Cells producing other immunoglobulins (e.g., γG_{2b} or γG_1) which are determined by other loci on the same chromosome (e.g., Ig-3b and Ig-4b) will not be affected by the suppressing antiserum since it does not react with these proteins. Since γG_{2a} globulins form less than 50% of the total immunoglobulins found in mouse serum, it is reasonable to expect that in a suppressed mouse there will be a sizable population of unaffected cells with the "b" type chromosome active.

Once the suppressing antiserum disappears, any cells from the above population which shift and de-repress the Ig-1b locus will now survive. Some will go on to be stimulated to further differentiation and the production of large amounts of Ig-1b globulin. The deficit of Ig-1b cells caused by the period during which no cells were able to differentiate into full Ig-1b production will, however, remain and be evident as a decreased rate of Ig-1b production for a long period.

In contrast, in rabbits the antigens with which the suppressing antiserum reacts are present on the several classes of immunoglobulins (γG, γA, and γM) comprising more than 90% of rabbit immunoglobulins (13). Therefore most, if not all of the stem cells with the active chromosome carrying the affected allotype would be removed. When the suppressing antiserum disappears, very few cells would be available then to shift and repopulate, leaving a more or less permanently allotype-suppressed rabbit as the result.

This model would also explain why suppressed homozygous mice more quickly recover from suppression than do heterozygous mice, since both chromosomes in the homozygote would yield cells capable of shifting to production of the suppressed allotype.

It is unfortunate that as yet no genetic linkage between immunoglobulin genes and other known genetic markers has been established, as such markers could allow a direct test of the analogy with X-chromosome inactivation. Perhaps though, no important recognizable genes other than immunoglobulins exist on the immunoglobulin chromsome, and therefore linkage will never be found.

One other finding brought to light incidentally in this work is perhaps of some biological significance and should be noted. The tendency of animals within a litter to be more similar with respect to the age at which they reach the threshold of 1g-1b detection than a group drawn at random from the population is puzzling. Since all animals within a group are genetically homogeneous, and since all parents are

also genetically homogeneous, it is difficult to see why offspring are clustered within litters rather than being distributed over the time of response of the whole population. It may be that Ig-1b level reflects the degree of response to environmental antigens, and therefore that animals sharing the same microenvironment, i.e., mother, cage, etc., are more likely to respond similarly, but the similarity of this finding to the inter-litter variation remarked on by Simonson (14) and others (15) suggests that perhaps the answer is related to prenatal effects on development of the immune system.

SUMMARY

In the mouse, antibody directed against an immunoglobulin allotype, Ig-1b, passed from mother to offspring or injected into neonates, suppresses synthesis of immunoglobulin carrying Ig-1b. In allotype homozygotes as well as hetero-zygotes the allotype suppression is manifested both by a delay of several weeks in attaining initial detectable allotype levels and a reduction in allotype level continuing into adulthood. A possible mechanism for the differentiation of the immune system consistent with both the kinetics of suppression reported here for the mouse and the comparatively longer lived and more complete allotype suppression described for the rabbit is discussed.

Evidence for a strong intralitter (as opposed to interlitter) correlation of age of onset of immunoglobulin allotype synthesis is presented.

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