QUANTITATIVE STUDIES OF THE ADOPTIVE IMMUNOLOGICAL MEMORY IN MICE

I. An Age-Dependent Barrier to Syngeneic Transplantation

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Quantitative studies on antibody production require the simultaneous control of many variables, e.g. cell number, available space, antigen concentration, in order to yield interpretable results from in vivo immune reactions. For these reasons several laboratories have utilized cell transfer in allo- and syngeneic combinations to investigate humoral antibody production, delayed hypersensitivity, and homograft reactivity: the subject has been extensively reviewed by Cochrane and Dixon (1). One serious limitation of the transfer system has been the immunological competence of the host animal, which would lead to cytotoxic action against the transferred cells in the case of allogeneic combinations (2), and to confusing additional reactivity against the test antigen in syngeneic combinations. To circumvent either difficulty lethal irradiation of the host, or utilization of newborn recipients have been adopted. It is evident that these measures would not allow a prolonged observation of the activity of transferred cells, as the recipients would either die due to radiation or (re)gain with time their undesired competence.

The main object of the present study was to establish a model system in which immunological memory could be measured in a known number of cells and for periods up to 6 months. The above mentioned limitation was avoided by selecting an antigen, human serum albumin, which has been found (3) to be almost devoid of immunogenic potency when injected over a large dose range in aqueous solution into mice. In contrast, when it is injected with Freund's adjuvant, the same protein is able to elicit a primary response and to "prime" immunocytes, to produce a secondary response at their next contact with the antigen in any form. Thus, if primed cells are transferred to a nonprimed mouse of the same genotype, they are operationally distinct from the cells of the new environment in a qualitative rather than quantitative manner: they are the only cells capable of responding in a detectable way to the fluid antigen stimulation. The possibility of studying in ideal conditions, the history of this "pure" secondary response is indeed adequate for the present purpose. This first paper of the series describes a convenient way to determine the titers of antisera by the Farr technique, and reports the phenomenon by which the functional implantation of competent cells in normal syngeneic mice is severely impaired. This "barrier"

is radiosensitive and rises gradually during the first 2 months of life of the recipient.

Materials and Methods

Mice.—A.SW and (A × DBA) F_1 animals of the same sex, bred in this Institute, were used in syngeneic donor-recipient combination. Since a detectable reaction against the Y-linked antigen was observed in the present as well as in other similar experiments (4), male cells were never transferred into female recipients. The donors were injected subcutaneously with 5 mg human serum albumin (HSA) in Freund's adiuvant at the age of 3 to 4 months. They were utilized 40 days to 6 months thereafter. The recipients were used either as "adults" (2 to 4 months old) or at the age specifically indicated in the description of individual experiments.

Irradiation Conditions.—A Siemens X-ray machine was the radiation source. The conditions were: 190 kvP, 15 ma; inherent filtration, 1.5 mm Al; added filtration 1.0 mm Al plus 0.5 mm Cu. The mice were introduced into separate loculi of a circular perforated Lucite container placed at 50 cm distance from the source. The dose rate at the target was about 100 R per min. The dose delivered was measured continuously by a Philips integrating dosimeter whose ionization chamber was fitted in one loculus of the animal container. When not otherwise indicated, the "standard radiation dose" delivered was 500 R.

Spleen Cell Transfer.—Donor mice were sacrificed by decapitation and the blood collected for antibody determination. The spleen was gently teased with two pairs of very fine forceps, keeping the organ submerged in Eagle's minimal essential medium without serum. The cell suspension obtained was passed several times in a 5 ml syringe fitted with an 18-gauge needle, then filtrated through a 200 mesh/in. stainless steel cloth. One spleen yielded an average of 1.6×10^8 nucleated cells, more than 90% of which remained "viable" as judged by the dye exclusion test. The donor cell suspension was kept at 4°C and injected within 30 min into the tail vein of the recipients, or intraperitoneally (i.p.) into newborn recipients.

Antigen.—Chrystalline HSA, batch 7749 from the Nutritional Biochemical Corporation, Cleveland was used throughout the experiments to immunize donors and to challenge recipients. This HSA preparation was shown to give a single precipitation line in immunoelectrophoresis when confronted with either anti-HSA or with anti–normal human serum antibodies. For donor immunization, equal parts of a 20 mg/ml solution and Difco complete adiuvant (Difco Laboratories, Detroit) were thoroughly mixed and injected subcutaneously in aliquots of 0.5 ml/mouse to two sites of the abdomen. Challenge of recipients consisted in an i.p. injection of 0.2 ml of a solution of HSA in saline. The standard challenge dose was 0.1 mg/mouse, given shortly (within 1 hr) after cell transfer. This dose failed to elicit any primary response from adult intact nonirradiated mice. For the titration of antibodies, ^{131}I-labeled human serum albumin B.P. was obtained from the Radiochemical Centre, Amersham, England.

Sera.—Recipients were bled on day 10, 20, and 30 after having received the cell transfer and challenge, by puncturing the retroorbital sinus with a Pasteur pipette. 2 or 3 hr after clotting at room temperature individual sera were separated by centrifugation and kept frozen at −20°C until the moment of titration.

Serology.—The antigen-binding capacity (ABC) of the sera was determined by the technique described by Farr (5) to which several modifications were applied. Essentially, a fixed amount of I^{131} HSA (0.25 μg in 0.25 ml) was mixed with an equal amount of test serum, diluted in borate buffer at pH 8.2, containing 10% normal mouse serum (NMS). Standard dilutions employed were 1:5, 1:10, 1:25, 1:50, 1:100, 1:250, and 1:500. Contact between antigen-antibody was allowed for 30 min after which 0.5 ml saturated ammonium sulfate (SAS)

was added to each tube; after 60 min the precipitate was separated by centrifuging at 4900 *g* for 20 min and washed once in 1 ml 50% SAS in borate. All operations were carried out at room temperature. The final precipitate was dissolved in 1 ml borate buffer and counted in a Tracerlab well type scintillator. Each experiment had the following standards: (*a*) two positive controls consisting of tubes which received the normal amount of albumin and buffer but were not processed with SAS; and (*b*) two negative controls for the nonspecific albumin binding or trapping, consisting of albumin plus NMS-buffer which was regularly processed with SAS. The maximum nonspecific binding allowed was 4%. Usually 1.5 to 3% precipitation was found in the negative control tubes. The determination of the ABC, in terms of μg-bound antigen per ml undiluted serum, was calculated from the fraction of albumin precipitated by the SAS when preincubated with a given serum dilution. This fraction, called fb, corresponds to the amount of albumin which is bound when 1 ml of a given serum dilution is incubated with 1 ml of buffer containing 1 μg of albumin. The details of these calculations, which were standardized through a set of preliminary experiments, are described in the first section of the Results.

RESULTS

Antigen binding as a function of antibody concentration.—The aim of the experiments described in this section was to establish a reliable way of determining the titer of a given serum by the Farr technique. The fraction of ^{131}I-labeled HSA bound by an aliquot of antiserum depends on the concentration of antibody molecules as well as on their types. The latter parameter, first observed by Farr himself (5) can be disregarded in the present study, since both donor and recipient's sera were shown by chromatographic separation on Sephadex, to contain exclusively 7S antibodies, as expected for a late primary and a secondary response. The plot of the fraction of albumin bound (fb) by antisera of different strengths against serum dilution yielded roughly parallel S-shaped curves. Sigmoidal curves are inconvenient if one wishes to calculate the ABC of an antiserum using a small number of dilutions but without losing accuracy. Thus the next step was to transform them into straight lines. This was accomplished to a satisfactory degree by using Von Krogh's empirical equation, i.e. by substituting fb with the expression fb/(1 − fb). Log [fb/(1 − fb)] was plotted against the concentration (expressed as log μl/ml) for several antisera (Fig. 1). The resulting functions were linear and parallel over about two log units, corresponding to fb values from about 10 to 75%. Within these limits they could be described with the general formula

$$Y = a + 0.75\,X \tag{1}$$

where $Y = \log \mu l/ml$, X, $= \log fb/(1 - fb)$, 0.75 is the slope, and a is the intercept characteristic for each serum. This intercept expresses the logarithmic concentration of the serum when $\log [fb/(1 - fb)] = 0$; $fb/(1 - fb)$ is then $= 1$, and $fb = 0.5$: it corresponds to the log μl undiluted antiserum necessary to bind 0.5 μg HSA. The intercept a is readily calculated by applying formula (1) to any fb value observed with a given serum, provided that it is between 10

and 75%, and is expressed in log μl in 1 ml of serum dilution:

$$a = \log \mu l/ml - 0.75 \log [fb/(1 - fb)] \quad (2)$$

To obtain the ABC (μg HSA bound by 1 ml of undiluted antiserum), the num-

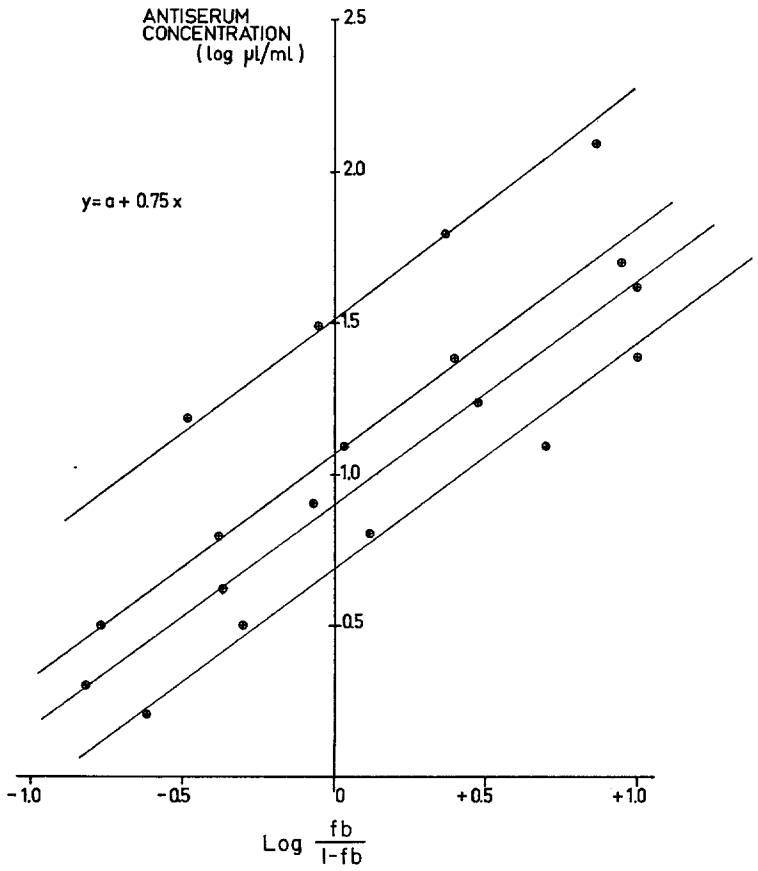

FIG. 1. Linear relationship between log antiserum concentration and log fb/(1 − fb), obtained by titrating 4 different antisera. Fb is the fraction of HSA bound by antibody in the reaction tube. The slope of the curves is 0.75. The intercept at log fb/(1 − fb) = 0 represents the antiserum concentration necessary to bind 50% of the HSA present.

ber of μl in 1 ml of undiluted serum (=1000) should be divided by twice the number of μl necessary to bind 0.5 μg HSA, or 50% of the albumin present. In log terms this is written:

$$\log ABC = 3.000 - (a + 0.30103), \quad (3)$$

or by approximation

$$\log ABC = 2.700 - a$$

The accuracy of determining the ABC of an antiserum by this method was tested by performing 6 parallel independent titrations on the same sample. This was repeated with two pools of antisera in serial dilutions. The mean log ABC and its 95% confidence limits, calculated from the *fb* of a series of serum con-

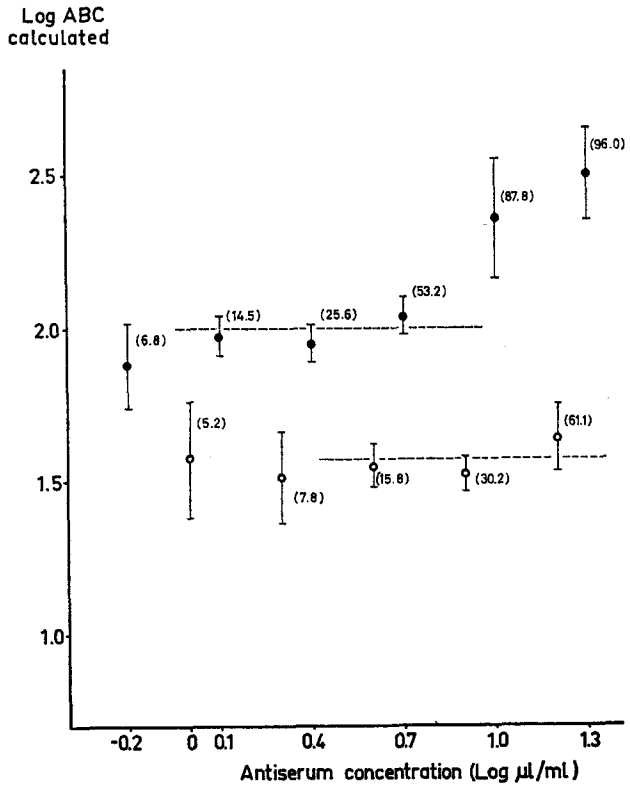

FIG. 2. Calculation of the titer (log ABC) of two antisera by the formula described in the text. Each point is the mean log ABC obtained from 6 independent dilutions of the same serum, ± the 95% confidence limits. Only the values calculated from per cent binding 10 to 75 are considered reliable. The dotted lines show the best estimates of the titers. In parenthesis is the mean per cent albumin bound at each dilution of antiserum.

centrations are shown in Fig. 2. The results confirm that the useful range to calculate ABC is limited to serum concentrations binding between 10 and 75%. Within these limits the mean titers obtained are statistically not different from each other and are subject to comparable variation. Outside these limits, variation tends to increase at both ends, while the calculated titer for fractions of HSA bound in the order of 85 to 95% is markedly higher as well.

Table I shows the individual data from the titration of the pool with the higher ABC. The distribution of the individual log ABC values is quite narrow

TABLE I

Test of the Accuracy of ABC Determination

Serum concentration	HSA binding (fb × 100)		Log ABC calculated		ABC geometric mean ±95% confidence limits
	Individual tubes	Mean	Individual tubes	Mean and SD	
μl/ml					
10	90.1 97.0 93.4 96.3 93.2 99.0	87.8	Out of useful range		—
5	51.5 49.2 61.0 54.2 51.4 51.9	53.1	2.010 1.990 2.140 2.057 2.019 2.017	2.040 s = 0.07 $s_{\bar{x}}$ = 0.03	109 ± 14
2.5	28.6 24.8 16.3 22.2 26.3 35.6	25.6	2.003 1.939 1.777 1.892 1.965 2.112	1.950 s = 0.07 $s_{\bar{x}}$ = 0.03	89 ± 15
1.25	14.4 14.3 13.0 10.6 9.6 14.8	12.8	2.021 2.019 1.982 1.910 1.872 2.041	1.974 s = 0.07 $s_{\bar{x}}$ = 0.029	94 ± 14
0.62	6.7 6.3 7.2 8.1 6.6 6.0	6.8	Out of useful range		—
0.00	2.0				

within the useful range (in this instance 8 to 61% binding), the percentual standard deviation of the distributions being less than 4.

The Adoptive Response.—Several characteristics of the adoptive secondary response against albumin have been described by Dresser (6) and by Mäkelä

and Mitchison (7, 8). In agreement with the data of these investigators, the results from preliminary experiments in the present study are the following. After transferring 10^7 presensitized spleen cells to irradiated syngeneic recipients and challenging them with a small dose (0.1 mg) of fluid HSA, the antibody response of these spleen cells (*a*) rose at a high rate during the first 10 days, reached a peak between 10 and 20 days, and decayed slowly thereafter;

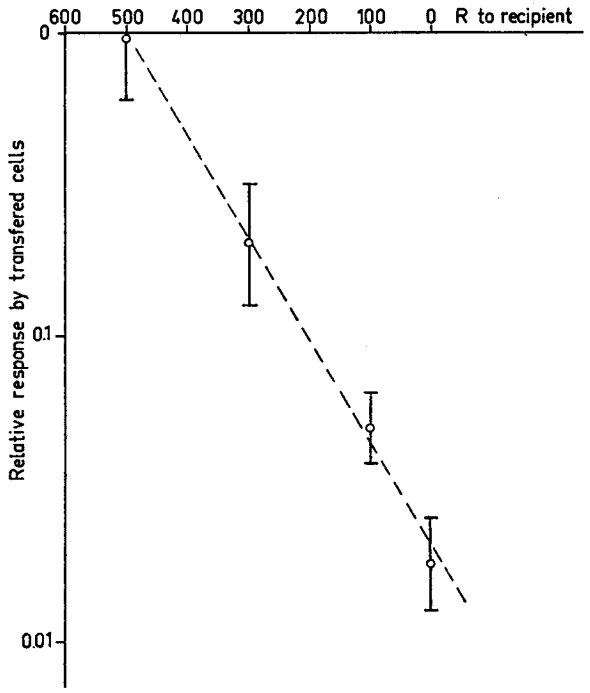

FIG. 3. Radiosensitivity of the barrier to syngeneic transplantation. The relative antibody response of 10^7 transferred spleen cells is plotted (ordinate) against decreasing doses of X-rays administered to the recipient.

(*b*) was about 200 times more efficient than the response of unchallenged cells, and (*c*) was 50 to 100 times more efficient than the response of cells transferred into nonirradiated adult recipients of the same strain. The fact, evident from observation (*c*), that donor cells were functionally impaired as if they had encountered a barrier when transferred into intact adult recipients was quantitatively studied in a series of experiments. These were designed to answer the following questions concerning the importance of the recipient environment in the transplantations of competent cells: (*a*) how radiosensitive is the "barrier"; (*b*) could it be based on recipient's immune reactivity against donor cells

prompted by an unexpected residual heterozygosis in the inbred strain used; and (*c*) is it dependent on the age of the recipients?

Impact of irradiation of the recipients.—To measure the effect of X-irradiation of the host on the function of antibody forming transferred cells the following experiment was performed. Groups of prospective recipients were exposed to 600, 500, 300, 100, and 0 R. Within 1 hr they were transferred 10^7 syngeneic spleen cells from a single donor pool. The challenging HSA injection was administered immediately thereafter. The results are shown in Fig. 3. The relative

TABLE II

Test for Possible Homograft Reaction Against Transfer Cells

Pretreatment of recipients	Log ABC individual	Mean	"Student" *t* test
None	0.714		
	0.919		
	1.112		
	0.825	1.022	
	1.462		
	1.379		
	0.844		
			0.630 (*t* value for 13 degrees of freedom = 2.160)
Intraperitoneal injection of 2×10^7 irradiated syngeneic cells	0.951		
	0.923		
	1.171		
	0.749	0.939	
	0.837		
	1.186		
	0.750		

antibody response in each group of mice is plotted on a semilog scale against the X-ray dose administered to the recipients. The antibody level found in 600 R recipients was taken as reference point=1, as higher doses do not further improve the conditions for the transferred cells to function. The effect of 500 R was not significantly different from the reference dose, while an increasing impairment of antibody production was observed with 300 and 100 R. These doses allowed about 20 and 5% of the response, respectively, while 1.5% was found in nonirradiated mice.

Test for Possible Homograft Reactivity.—The strains of mice used in these experiments are highly inbred and they are checked at intervals for intrastrain acceptance of skin grafts. However, in view of (*a*) the possible different sensitivity of skin and spleen cell graft rejection tests (2), (*b*) the possibility that

lymphoidal cells might carry antigens which are not expressed on cells of a different lineage (9), (*c*), the recently reported high incidence of spontaneous mutations at histocompatibility loci (10), and (*d*) the parallelism at an interval of 500 R, of the radiosensitivity profile of the allogeneic transplantation barrier

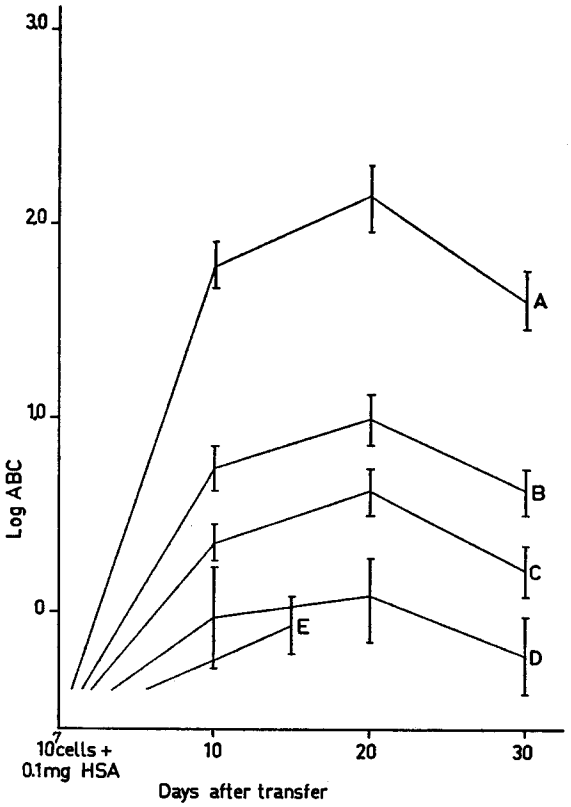

FIG. 4. Antibody response of 10^7 donor spleen cells transferred: A, into 500 R irradiated; B, 30 days old; C, 39 days old; D, 66 days old; and E, 120 days old, nonirradiated recipients. Groups of 6 to 10 mice. Plotted are mean log ABC ± 95% confidence limits.

as measured in a system closely resembling the present one (11) and of the syngeneic barrier illustrated in Fig. 3, the existence of any degree of immune reactivity of the recipients against the donor cells had to be carefully excluded.

This was done by transferring immune spleen cells in the usual way to intact recipients which had been pretreated, 1 wk previously, with 2×10^7 cells of a pool from several syngeneic animals. These cells were irradiated with 3000 R in vitro, and inoculated i.p. The results, in Table II, show the titers obtained in the experimental groups, as compared with the controls which were not pre-

sensitized. There is a clear demonstration that homograft reaction against the hypothetical antigenic specificities of the donors plays no role in this model system, or else preimmunized recipients would have reacted more strongly, with a resulting lower anti-HSA titer in this group.

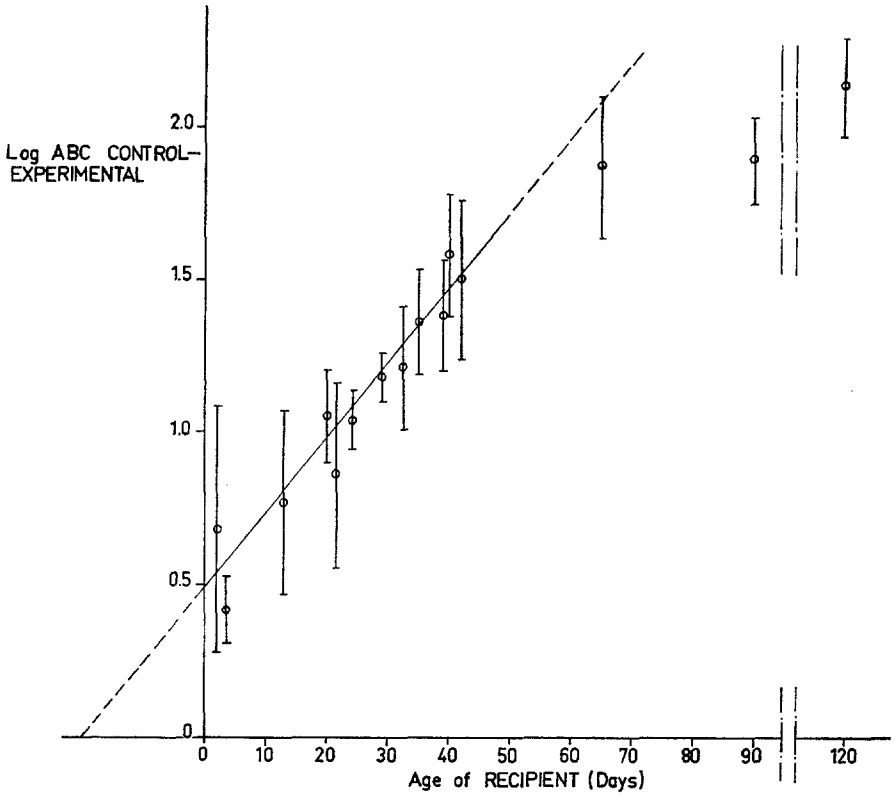

FIG. 5. Increase with recipient's age of the barrier to functional transplantation of donor cells. The relative inhibition of donor cells (log ABC in irradiated controls − log ABC in experimental groups) is plotted against the age of recipients. Each point is the mean of 5 to 10 observations, ±95% confidence limits. The fitted regression line from 2 to 65 days of age is expressed by the formula $Y = 0.493 + 0.024\ X$. For $Y = 0$, $X = -20.9$ days.

Age Dependence of the Barrier.—It was observed that irradiated recipients of varying ages from 20 days to over 4 months provided an equally good environment for the production of antibodies by donor cells. By contrast, when the same pool of donor cells was transferred to groups of *intact* mice of different ages the ABC of their serum dropped gradually from 50% of the irradiated recipients (control), in newborns, to less than 2% in adults (60 to 80 days old).

The results of one experiment are shown in Fig. 4, while the complete series of experiments involving about 150 experimental recipients and 100 controls (irradiated recipients), is illustrated in Fig. 5. In this graph (Fig. 5) the barrier to functional "take" of donor cells is represented as the difference log $ABC_{control} = ABC_{experimental}$ and is plotted against recipient's age. Each experiment usually included one or two experimental and one control group, with 5 to 10 animals per group. The individual log ABC values obtained at each bleeding (10, 20, and 30 days) were subtracted from the control mean log ABC of the corresponding bleeding. Each point on the graph in Fig. 5 is the mean of the pooled values from the three bleedings. The 95% confidence limits were calculated as follows: $t_{\times}(SD)/n$ where t is Student's value corresponding to $p=0.05$ for $n-1$ degrees of freedom, n is the number of animals in the experimental group, and SD is the mean of the standard deviations computed for each bleeding.

A possible error, due to the change of blood volume with age, was avoided by always using "irradiated control" mice of the same age as the experimental animals.

Despite considerable variation within experiments the resulting function shows a linear rise over the first 2½ months of age, and reaches a plateau thereafter. The regression line, fitted to the data from 2 to 65 days of age is expressed by the formula: $Y = 0.493 + 0.024\,X$. By extrapolation, the barrier (Y) is 0 at age -20.9 days; this time corresponds to the very beginning of the embryonic life of the mouse.

DISCUSSION

The method for calculating ABC described in the first section of this paper is useful as it readily allows extrapolation from a wide range of binding of HSA to the corresponding 50% binding. Thus, the titer of an antiserum can be obtained with satisfactory accuracy from a single tube, provided the binding is between 10 and 75%. The results are comparable or better than other methods of calculation proposed (5), and the variation within repetitions is small.

The existence of an impairment of the functional activity of antibody-forming cells transferred into intact syngeneic recipients, and the abrogation of this state by previous exposure of the recipient to radiation, has been observed by Dresser (6) who measured the adoptive response of mice against BSA by the accelerated elimination of ^{131}Iodinated antigen from their blood stream, and by Weiler, who followed the antiphage titer in mice injected with syngeneic immune ascites cells (12). This phenomenon could be quantitated in the present transfer system, although it is not known whether the same number of cells are implanted in intact and irradiated recipients but are allowed a different rate of antibody production, or a different number of cells "take" in the first place. The total body radiation doses (500 to 600 R), which abrogate the barrier are in the same order of magnitude of those necessary to inhibit for a limited period

a primary immune response of intact mice to heterologous antigen; such a response in turn has been shown to be as radiosensitive at a cellular level as any rapidly dividing cell population, both in vivo (13, 14, 11) and in vitro. Thus these data are compatible with the existence of a competition between the inoculum and the recipients' own lymphoidal cells. It is known that there is an upper limit in the number of lymphoidal, and hence competent cells in the intact animal. Radiation, by destroying most of the recipients dividing cells, would make "space" for the functional implantation of the donor cells. It should however be pointed out that the meaning of such concepts like "competition" and "space" as used here is still vague, since information is wanting with regard to the possible importance, as regulatory factors, of (*a*), humoral versus cell contact limitation of lymphoidal growth, (*b*) specific or nonspecific gamma globulin feedback on antibody production, and (*c*), central versus microenvironmental stimulation/depression of mitosis, which is regarded as necessary for the building up of a complete immune response.

To extrapolate from the data here obtained in the transfer model to the regulatory mechanisms of the intact mouse, one should assume that transferred and autochthonous cells being derived from the same original genotype share the same fate. This seems to be a reasonable assumption: hence a working hypothesis of cellular homeostasis should certainly cope with the developmental aspect shown by the present experiments, i.e. the growth of repressive forces during a period beginning in early embryonic life and ending at the full maturation of the animal.

The results with newborn or very young mice confirm the difference between the rabbit and several other laboratory animals as far as the ability of the newborn to support an immune response by transferred adult competent cells is concerned (1). A number of parameters of the ontogenic development of the mouse and particularly of its lymphoidal tissue show a gross parallelism or time coincidence with the rise of the barrier to syngeneic implantation of immunologically competent cells. For instance Metcalf's data on thymus (16) and lymph node (17) growth, Makinodan's measure of the relative efficiency of the primary response by spleen cells (18), and Wigzell's estimate of the increase of hemolytic plaque-forming cells in the spleen (19), all present a maximum growth rate between birth and the age of 2 months, a period which also includes the main weight gain of the animal. However, a more quantitative comparison of these parameters reveals that in the young mice the doubling time of body, spleen and thymus weight is about 2 wk, while the potential antibody-forming cells double in 1 wk or less. The doubling time of the "barrier" calculated from the present data is about 12 days. This suggests that the barrier is not directly correlated with the actual number of mature immune cells present in the animal, but is rather an expression of a more general regulation of the lymphoidal system. The interpretation of this phenomenon as central is favored in view of the

results of current experiments in this laboratory, which indicate that 1-yr-old irradiated recipients are a significantly worst environment for the donor cells than their 3-month-old similarly treated counterparts. Since it is known that in the senescent mouse the content of ab-forming cells in the spleen declines, while the body and spleen weight do not (18, 19), one should have expected an equal or better take in old mice if the barrier was due to simple competition among immunologically competent cells.

SUMMARY

Antibody-forming cells suspended from a mouse spleen and transferred to intact animals of the same genotype face a barrier which severely affects their capacity to implant and/or to function.

This phenomenon was quantitatively studied in a model system which, utilizing the immunogenic properties of human serum albumin in mice, allows the secondary response of the transferred cells to be followed without interference from the host's own reactivity. The barrier to syngeneic transplantation was found (*a*) to be radiosensitive (500 R X-rays to the recipient abolishes it and insures optimal functional conditions to the donor cells) in the same order of magnitude of other mammalian systems involving rapidly dividing cell populations, and (*b*) to depend upon the age of the recipient: its linear rise is documented from birth time (when $\sim$ 50% of the maximal immune capacity of the transfer is expressed) to the age of 2 months ($\sim$ 1%). The significance of these findings to the immune response and to cell growth and differentiation is discussed.

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