QUANTITATIVE STUDIES OF THE ADOPTIVE IMMUNOLOGICAL MEMORY IN MICE

II. Linear Transmission of Cellular Memory

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When an animal is reexposed to an antigen a given time after the first contact, it produces a secondary response. The final number of antibody-forming cells engaged in the secondary response is 100–150 times higher than in the primary (1). This difference cannot be explained on the basis of two types of cells involved, higher amount of antibody produced per cell, or higher cell division rate (2, 3). It follows that the number of cells which the antigen can trigger to initiate the characteristic division-differentiation-synthesis process increases drastically after primary immunization.

In this paper immunological memory is defined as the *persistence* of these cells at high level for periods up to the life span of the animal. Two basic mechanisms could account for it: (*a*) the presence of a cell line along which the information for initiating a specific response upon challenge is transmitted from mother to daughter cells, or (*b*) the recruitment of cells previously not responsive; this process could be mediated either by the antigen or by some other type of informational molecule. Some difficulties of assessing the importance of these mechanisms in the intact animal have been circumvented by developing a system, described in the first paper of this series (4), which permits measurement of immunological memory in isolated, adoptively transferred cell populations. Since the antigen used (human serum albumin) is not immunogenic for the mouse, when in soluble form, the interference of the recipient's own immune capacity is avoided, and the system provides a means of distinguishing the linear transmission from the recruitment mechanisms. In this paper several parameters—e.g. cell dose, time, antigen dose, and irradiation effect— are studied, and a profile of the decay of immunological memory is presented.

Materials and Methods

Mice.—A.SW/KL and (A × CBA)F₁ animals were used throughout the experiments, in syngeneic donor-recipient combination. In each experiment mice of the same sex were used. Donors were immunized at 3 months of age and killed 1–6 months later. Recipients were 2–4 months old at the time of the transfer.

Irradiation Conditions.—A Siemens X-ray machine was used, under the following conditions: 190 Kv peak, 15 ma; filtration: inherent, 1.5 mm Al; added, 1.0 mm Al + 0.5 mm Cu.

The dose rate, at 50 cm from the source, was about 100 R/min. The standard dose delivered to prospective recipients was 500 R.

Spleen Cell Transfer and Antigen.—These procedures have been described in detail in the first paper in this series (4).

Serology.—Anti–human serum albumin (HSA) antibodies were titrated by the Farr technique, with the procedure described earlier (4). The calculations of antigen-binding capacity (ABC) of an antiserum, expressed in micrograms of HSA bound per milliliter of undiluted serum, was done by extrapolating, from the serum concentration capable of binding a certain fraction (between 10 and 75%) of the ^{131}I-labeled human serum albumin present in the test tube, the concentration, expressed as log microliters per milliliter, which would bind 50% of the antigen. This extrapolation a was obtained by the formula

$$a = \log c - 0.75 \log [fb/(1 - fb)]$$

where c is the serum concentration used, expressed in microliters per milliliter, and fb the fraction of albumin bound. The constant 0.75 was determined with a final antigen concentration of 0.5 μg/ml in the reaction mixture. The log ABC of the undiluted serum was then calculated by subtracting the value a from the constant 2.698. Since these calculations, however simple, are time-consuming, they are conveniently performed by computer, together with the statistical treatment of the data.

RESULTS

The Relation between Transferred Cell Number and Antibody Produced.—In order to define the conditions of the present system, groups of irradiated mice were given 2×10^6, 6×10^6, or 2×10^7 spleen cells from the same donor pool, and were challenged immediately with 100 μg fluid HSA. The determination of the ABC of their serum was done 10, 20, and 30 days after challenge. The results of several experiments are shown in Fig. 1, where the 10 day titer (ABC) is plotted against the number of transferred cells on a log/log scale. The log titer increases linearly with the increase of the log of the cell number. The slope is about 1.8. A log/log regression with a slope greater than 1 corresponds to an accelerating curve in arithmetical scale. In this case, by increasing 10-fold the number of cells transferred, the titer rises 60-fold. A systematic study of the phenomenon, which is considered here as a "premium" activity of the larger inocula, reveals that the only condition where the premium is not found is when spleen cells are transferred to nonirradiated, 1 month old recipients (Fig. 1), a situation where the expansion of transferred cells is depressed (4). A certain number of the transferred cells might be diverted from potential antibody formation to the fulfillment of the physiological functions necessary to keep a severely irradiated mouse alive (5), a phenomenon which would affect the smallest inocula more severely. This possibility was tested by mixing immune and nonimmune cells in various proportions while keeping the total number of injected cells constant. The results, also included in Fig. 1, show that this operation did not influence the premium.

The "Premium Effect" in Relation to Antigen Dose and Time of Assay.—The observed picture would be easily explained if optimal stimulation occurred at

different antigen doses for different cell numbers. This possibility was tested by performing two experiments. The first one consisted in giving the challenge in vitro, incubating the donor spleen cell pool with HSA (3×10^8 cells and 100 micrograms of HSA per milliliter) for 1 hr at 37°C, washing once, diluting to the desired concentration, and injecting different cell doses into irradiated recipients. The results were not different from the previous ones (Fig. 1).
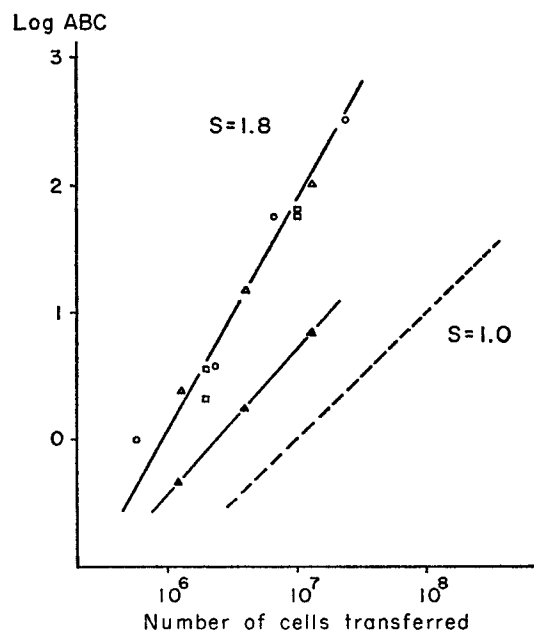

Fig. 1. Efficiency of adoptive antibody production relative to the number of transferred spleen cells. Log ABC 10 days after challenge is plotted against cell number on a logarithmic scale. In vivo challenge, △; in vitro challenge, ○; cell number kept constant by mixing immune and "normal" cells (two separate experiments), □; cells transferred to 1 month old, nonirradiated recipients, ▲. Eight to 10 animals per group. The broken line, given for reference, has a theoretical slope of 1.

The second experiment consisted in a complete mapping of cell and antigen doses. Each inoculum of 2×10^6, 6×10^6, and 2×10^7 immune cells was given to eight groups of recipients, and each antigen dose of a series ranging in 10-fold steps from 10^{-4} to 10^3 µg was administered to three groups of mice that had received the three doses of cells. The results of this experiment are shown in Fig. 2, where the peak titers, expressed as log ABC, are plotted against the dose of challenging HSA for each cell dose. The resulting curves are roughly parallel for most of their span; in agreement with Mäkelä and Mitchison (6), 10^{-3} µg is the minimum dose that elicits a secondary response detectable over

the residual primary titer produced in the absence of challenge. The substantial increase in antibody formed takes place between 10^{-3} and 1 μg challenge, with a rate approaching proportionality between antigen injected and
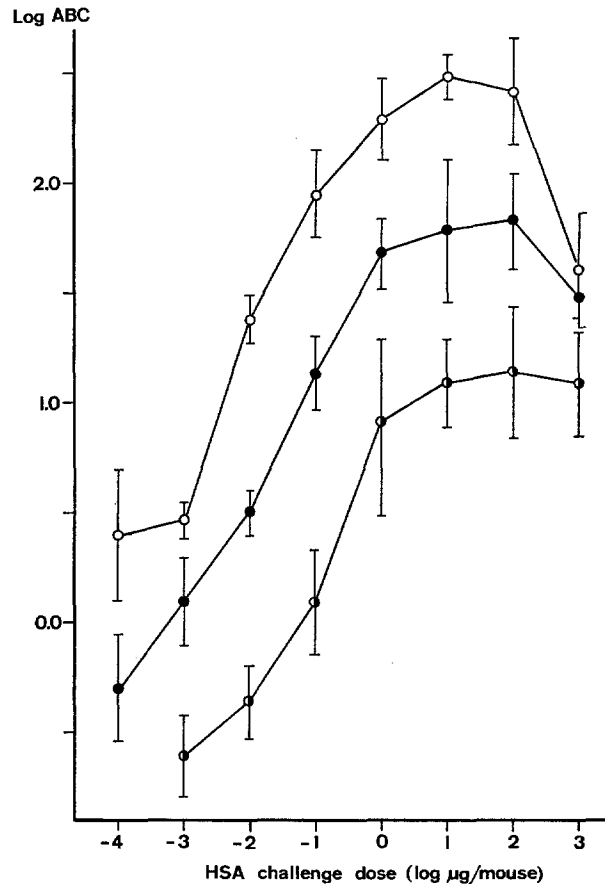

FIG. 2. Profile of secondary immune response of three cell numbers, relative to the dose of challenging antigen. Log ABC at peak level is plotted against log micrograms of HSA injected into the recipients after transfer. 2 $\times$ 10^7 transferred spleen cells, O; 6 $\times$ 10^6, ●; 2 $\times$ 10^6, ◑. Each point represents the mean of 8–10 animals. Vertical bars indicate the 95% confidence limits.

antibody synthesized. A plateau, indicating optimal stimulation of the transferred cells, is reached between 10 and 100 μg; by further increasing the challenge dose to 1000 μg, the effect on the three transfer cell doses is a depression of the plateau level. This paralysis effect on primed cells is particularly marked on the *highest* cell dose, where the actual antibody produced is less than 20%

of the amount produced upon optimal stimulation, while the activity of the smallest cell dose is almost unaffected.

The distance between the highest and the lowest curve in Fig. 2 is, with the exception of the points corresponding to 10^{-4} and to 10^3 μg challenge, always greater than 1 log unit on the ordinate. Since the two extreme cell doses considered here are 10-fold apart, this observation points out the consistency of the "premium effect" over the entire range of antigen doses used to elicit a secondary response, provided there is no antigen-induced paralysis.

TABLE I

Effect of Antigen Dose and Bleeding Time on the Efficiency of Antibody Production (ΔLog ABC/ΔLog Number of Cells Transferred)

Challenge dose of HSA/ mouse	10-day slope	S	df	P*	20-day slope	S	df	P	30-day slope	S	df	P
μg												
0.0001	1.384‡	0.209	17	<0.10	1.199	0.139	15	<0.20	0.369	0.185	16	—
0.001	1.289‡	0.129	20	<0.05	1.199	0.130	19	<0.20	0.893	0.109	19	—
0.01	1.773‡	0.109	19	<0.001	1.589	0.078	17	<0.001	1.546	0.134	18	<0.001
0.1	1.777	0.169	20	<0.001	2.336‡	0.122	20	<0.001	1.937	0.148	17	<0.001
1.0	1.442	0.279	18	<0.20	1.560‡	0.174	14	<0.005	1.517	0.182	14	<0.01
10.0	2.066	0.116	18	<0.001	1.356‡	0.283	13	<0.20	1.291	0.210	13	<0.20
100.0	1.485	0.153	20	<0.005	2.556‡	0.492	7	<0.02	1.067	0.090	15	0.50
1000.0	1.062	0.167	17	0.60	0.511‡	0.179	11	—	0.592	0.181	13	—

For each challenge dose the slopes were calculated from the regression lines fitted to 10-, 20-, and 30-day titers for 3 or 4 cell doses. For each slope the standard deviation (S), the degrees of freedom (df), and the probability level (P) are listed. Intercepts are omitted.

* Hypothesis tested: experimental slope = 1. Calculation: (slope − 1)/S = t. The result was compared with the t values for the corresponding degrees of freedom. The table used lists the following P values: 0.20, 0.10, 0.05, 0.025, 0.02, 0.01, 0.005, 0.001.

‡ Peak titer is reached at this bleeding.

Antigen dose has also an effect on the time interval between challenge and attainment of antibody peak level. The peak is near the first bleeding (10 days) with a challenge of 0.001 and 0.1 μg HSA, while it is at the second bleeding (20 days) with higher antigen doses. Table I shows in greater detail the time relationship among challenge, antibody peak, and relative efficiency per cell (in terms of titer/cell dose slope) for each antigen dose. The slope is greater than 1 in practically every instance, although there is a tendency of the "premium" to decrease after peak titer has been reached. The greatest premium (200-fold increase of antibody for 10-fold cell number) is found 20 days after the suboptimal challenge dose of 0.1 μg HSA.

The Secondary Response after Immediate or Delayed Challenge.—A series of experiments was performed to answer the following questions: (a) What is the magnitude of the secondary response of transferred cells challenged after a long sojourn in the syngeneic recipient, relative to that produced immediately

after transfer? (b) Do preirradiation of the recipients and inoculum size affect the kinetics of memory?

Immune spleen cells from the same donor pool were transferred to several groups of recipients, either exposed to 500 R or nonirradiated. The challenge (10 or 100 μg HSA intraperitoneally) was given to one group immediately after transfer, and to the other ones at different time intervals, e.g. 1 or 2 months or longer. The antibody titers in the serum of the recipients were
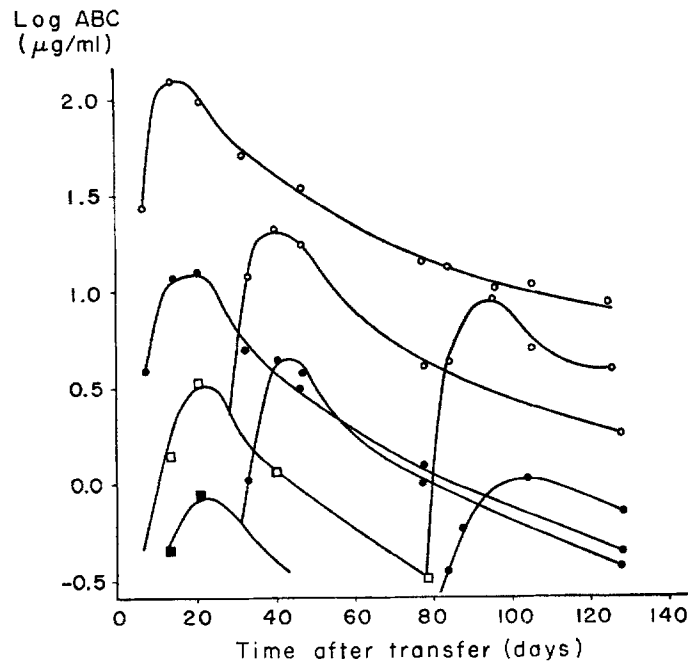

Fig. 3. Kinetics of the adoptive secondary response elicited immediately, 28, or 78 days after spleen cell transfer. Log ABC is plotted against time after transfer. 2 × 10⁷ cells transferred, ○ and □; 6 × 10⁶, ● and ■. Circles show titers produced after challenge with 10 μg HSA. Squares show titers produced without challenge ("residual primary response"). Each point is the mean of 8–10 animals.

followed from the day of transfer (for the groups receiving immediate challenge and for groups not challenged at all) or from the day of challenge (for the other groups).

One of these experiments, where two cell doses were transferred, is shown in Fig. 3. It has the following features: (a) Nonchallenged groups show an antibody titer which increases to reach a peak at about day 20 and then declines steadily. This titer, termed here "residual primary response," is directly proportional to the number of cells transferred. (b) The groups receiving the antigen immediately after transfer have the highest antibody titer, the time and shape of the peak being very similar to those of nonchallenged recipients, at a higher

level. In Fig. 3 this level is about 60 times greater than the residual primary response. In other experiments, a lower residual primary response was usually observed, and the secondary response had a gain factor of about 100. The peak titer in recipients to which different cell doses were transferred shows the phenomenon of "premium" at the higher dose. This is true also for delayed challenges. (c) The antibody peaks in recipients challenged at intervals after receiving the transfer grow lower with the increase of the interval between
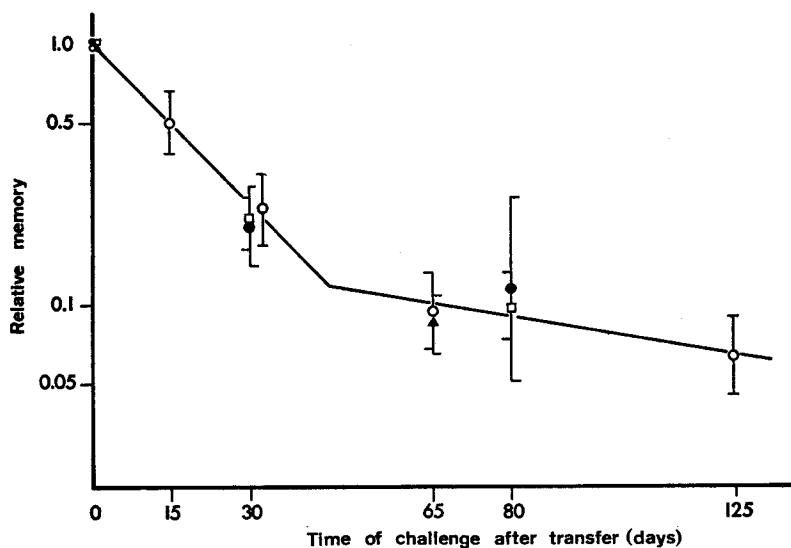

Fig. 4. Biphasic decline of immunological memory. The peak titer reached upon delayed challenge relative to titer reached upon immediate stimulation is plotted on a semilogarithmic scale. On the abscissa: time of challenge after cell transfer. Each point represents the mean of 5–10 mice. Vertical bars indicate the 95% confidence limits. The same key is used for data from the same transfer experiment.

transfer and challenge. The difference between challenge at time 0 and challenge at 1–2 months is greater than between 1 month and 3–4 months. This picture is true for all cell doses tested (Fig. 3) and also for the case when the spleen cells are transferred into nonirradiated young recipients, although their antibody-forming efficiency is lower, as has been described (4). (d) Recipients of spleen cells from donor mice either nontreated or treated only with Freund's adjuvant did not produce any antibody in response to the challenge.

The Measure of the Decline of Memory.—A profile of the immunological memory of the primed cells over several months is obtained by connecting the mean peak titers reached when the same number of cells from the same donor pool is challenged immediately vs. at given intervals after transfer. Data from several experiments, including those illustrated in Fig. 3, are combined in Fig. 4. With-

in each experiment the peak titers after delayed stimulation are expressed relative to the peak titer following the time 0 challenge, taken as 1.0. The profile shows a biphasic decline. The half-life of the hand-fitted slopes is 15 days during the first 40 days, and about 100 days during the following 3 months. To express the memory as the number of antigen-responsive cells present at any given time, it is necessary to correct for the nonproportionality of cell number and antibody titer. This is done by solving for x the equation $y = 1.8\ x$, which describes the relative titer (y) as a function of cell number (x). 1.8 is the mean slope determined experimentally. After correction, the two phases of decline of the potentially responding cell population have half-lives of 26 days and 190 days, respectively. There is a similarity between the decay curve of the memory and that of serum titers after the peak of a secondary or of a "residual" primary response is reached; this is evident if one compares Figs. 3 and 4. However, while the first curve is a direct measure of the secondary antibody-forming

TABLE II

Decline of Potential Immune Capacity of Normal and Irradiated Transfer Cells

Day of challenge after transfer	Control peak titer (log ABC)*	150 R peak titer (log ABC)*	(150 R ABC/ control ABC) × 100	(Control ABC/ control, time 0) × 100	(150 R ABC/150 R, time 0) × 100
0	2.11 ± 0.21	1.48 ± 0.30	23	100	100
30	1.35 ± 0.30	0.80 ± 0.14	29.5	17	21
80	0.99 ± 0.44	0.60 ± 0.16	40	7.7	13

* Mean ± 95% confidence limits.

potential of the transferred population, the second one is more complex, resulting from continued production, accumulation, and natural decay rate of the antibody.

The Tertiary Response.—A tertiary response was elicited by injecting mice, which had already been challenged at the time of transfer, with another standard dose of HSA. The results of these experiments showed that (a) when the tertiary stimulation was given during the first month after the secondary, no significant increase of the circulating antibody was obtained; (b) when the interval between the two challenges was 2–4 months there was a consistently significant rise in titer over the residual secondary titer, although (c) the peak of the tertiary response was not significantly different from that of the secondary response elicited at the same time, in a group that had received a transfer from the same cell pool, but had not been challenged at time 0.

The Radiosensitivity of Memory.—The radiosensitivity of memory was tested in the present system by studying (a) the effect of irradiation to the spleen cells, at the time of transfer, on their ability to build up an immediate vs. delayed secondary response, and (b) the effect of irradiation at the time of challenge of cells cultured for short vs. long periods in the recipients.

The first experiment was performed by exposing the donor cell suspension in vitro to 150 R prior to transfer into irradiated recipients; these, in separate groups, were challenged on days 0, 30, and 80 after transfer. Control groups received nonirradiated cells and were challenged at the same times. 150 R was chosen as the dose suppressing about 80% of the functional cells, according to preliminary experiments in this system, as well as in other transfer systems utilizing immediate secondary challenge.

Relative
titer

2.0

1.0

0.5

0.1

0.05

O   150   300   500
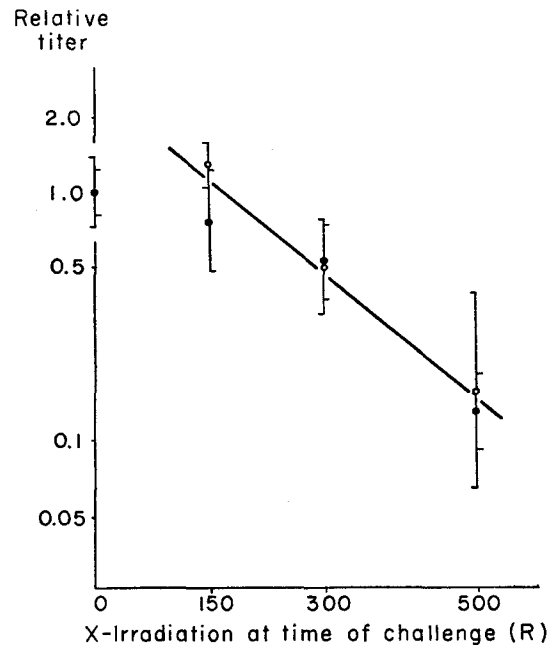X-Irradiation at time of challenge (R)

FIG. 5. Relative antibody production by transferred spleen cells when irradiated in the recipient, at the time of challenge. The residual titer is plotted against the radiation dose in R, on a semilog scale, the titer in the absence of irradiation being equal to 1. Transfer on the same day of irradiation and challenge, O; transfer 30 days before irradiation and challenge, ●. Each point represents the mean of 6–10 mice. Vertical bars indicate the 95% confidence limits.

The results are summarized in Table II, where the peak titers produced by irradiated and nontreated cells are given for each challenge time, as mean ABC ± 95% confidence intervals. The columns with the relative values essentially show that exposure to X-rays destroyed a fraction of the antibody-forming potential of the transferred cells, and that the decline of memory of irradiated and control cells is roughly parallel thereafter. There is no statistically significant evidence of long-term repair of the damage or any delayed manifestation of it.

A second experiment was designed to compare the effect of radiation on the "memory cells" at the time of challenge. Two series of groups of recipients were exposed to 0, 150, 300,

and 400 R X-rays. To one of the two series, donor cells had been transferred 1 hr previously, while the other had received a similar transfer 30 days earlier.

In Fig. 5 the results are expressed in terms of surviving activity at any given dose, relative to the activity (peak antibody titer) at 0 R. The picture reflects a complex phenomenon, since radiation given under these conditions has two potential effects: It kills part of the competent cells, but at the same time it enhances the activity of the survivors, as it destroys the so-called barrier to syngeneic transplantation (4), presumably by producing more space for population expansion. The resultant of these opposite effects can be predicted from the inactivation curves for antibody-forming cells (7) and for the "barrier" (4). As expected, the inactivation is less efficient than when spleen cells are irradiated prior to transfer; there is a considerable shoulder, and, in the case of the recently transferred cells, a tendency to overshoot, relatively to the nonirradiated controls (Fig. 5). Comparison of the two curves fails to show any significant difference in radiosensitivity between the freshly transferred and the in vivo cultured cell populations.

DISCUSSION

Four points are relevant for the interpretation of the present results, and should be discussed: (a) the accuracy of quantitation of the immune response, (b) the assumption that there is no recruitment of host cells in the adoptive immunity anti-HSA, (c) the possible cytokinetic model based on the memory decay picture, and (d) the significance of adoptive immunological memory for the anamnestic capacity of the intact animal.

We find that the serum titer of anti-HSA is not proportional to the number of immune cells transferred to an irradiated mouse: the increase in log titer (antigen-binding capacity titers are distributed log-normally) is ~1.8 for 1 log unit increase in cell number. This constant finding, although it does not prevent extrapolation from antibody in the circulation to active cells in the animal, is certainly unexpected. Proportionality was found by Makinodan et al. (8), working with particulate antigens—with the exception of the very early response—and by Mäkelä and Mitchison with soluble protein antigens (9). The contradiction between the latter report and the present one might be only apparent, since we used a lower range of cell doses: higher inocula saturate the system, thereby depressing the efficiency of antibody production per cell (1).

The mechanism of the "premium" phenomenon remains unexplained, after antigen concentration, absolute number of cells transferred, and time after challenge are excluded as possible causes. We are forced to postulate some sort of collaboration among antibody-forming cells; their physical vicinity in the case of high inocula would increase the probability of interaction. This in turn would enhance either cell division, rate of synthesis, and/or utilization of antigen. Along this line of reasoning one can imagine the formation of structures similar to germinal centers to be necessary for adoptive antibody formation.

Preliminary experiments indicate that anti-HSA response by small cell trans-
fers is enhanced by the presence of syngeneic cells reacting to an unrelated
antigen (Celada, unpublished data). This suggests a *nonspecific* collaboration.
Another possibility, which is presently being checked in our laboratory, is that
the *quality* of the antibodies produced by a big cell inoculum differs from that
produced by a small one, e.g., in antigen-binding efficiency.

There is considerable evidence that the adoptive response in the present
system is due solely to the activity of the donor cells and their descendants: (*a*)
No antibodies are found when the standard challenge dose of HSA is injected
into a normal mouse or a mouse to which nonimmune syngeneic cells have been
transferred. (*b*) The potential antibody-forming capacity of the transfer cells
is highly radiosensitive, since protein and RNA synthesis as well as antigen
disposal by phagocytes are more radioresistant (10, 11). This finding suggests
that mitosis is an essential step in the adoptive response. (*c*) Exposure of pros-
pective recipients to X-ray doses which are known to reduce drastically the
lymphoid cell number results in an enhanced adoptive response (4). The op-
posite effect would be obtained if the recipient's own cells played any important
part in it. The decline of memory, in the absence of self-renewal, also speaks
against horizontal transmission of information from the donor to the recipient.
We interpret it as a slow dying off (or diverging) of the competent cell line, with
a considerably long half-life (6 months, in the second phase). Our observation
time, when the age of the donors is also taken into account, is too short to
identify the memory decay with a recently studied phenomenon, the senescence
of the immune system (12).

The mechanism responsible for a *linear transmission of memory* could be
genetic or cytoplasmic: For instance, a "unit" of antigen could be passed along
from mother to daughter cell. The present data do not allow a discrimination
at this level, although recent experiments in this laboratory have demonstrated
that the half-life of HSA as an immunogen is on the order of 1 day in normal
mice.[1] The cytokinetics leading to the anamnestic response can also be visualized
in two alternative ways, which can be tested experimentally.

The One Cell Line Hypothesis (*A*).—Memory cells and actual antibody pro-
ducers comprise the same cell population. Its expansion is triggered either by
the antigen or by nonspecific stimuli, and is limited by the number of mitoses
a cell can undergo. The antigen acts also as an inducer of antibody production.
The expansion of the population of competent donor cells is the same in the
presence or the absence of antigen, the lethally irradiated recipient providing
the nonspecific stimulus for cell division. If the antigen is present at time 0,
antibody production rises and decays with the expansion and slow decay of the
transferred population. If the antigen is made available only later, the expansion
of the transfer will not be different, but it will not be reflected in antibody pro-

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[1] S. Britton and F. Celada. In manuscript.

duction; this function will only begin at the time of the injection of antigen inducer. At this very moment the cells will begin to produce antibody, without having to undergo further mitoses. If the challenge is given when the population curve is declining, the titer consequently will be lower than the peak reached with a time 0 challenge.

The Two Cell Lines Hypothesis (B).—The adoptive secondary response is the result of the activity of two cell lines, one deriving from the other. One, the "memory" cells, are stem cells which have been specifically determined or "selected out" at the time of the first contact with the antigen. They pass on, from mother to daughter cell through mitosis, the capacity to give rise to an antibody-producing cell line. The characteristic effect of the antigen is to produce the second cell line from the "memory" line. Since the memory line is not exhausted in this process, this event may take place through asymmetrical mitosis. The antibody-producing line undergoes rapid mitotic expansion and differentiation while producing antibody. These are the well-known features of the secondary response (13). The memory cell line sets the starting point for the "production line." Since the latter is assumed to have a limited number of divisions to undergo, it also gives the limit of final antibody peak at each time after the primary stimulation. Thus, the size of the peak titers at different intervals after transfer reflects the decline of the memory cells. There are two points which allow discrimination between (A) and (B): the radiosensitivity of the delayed vs. the immediate secondary response [according to (A) no mitosis would be necessary in a delayed response] and the influence of the secondary stimulation on the magnitude of the tertiary response. Both experiments are described in this paper, and the results are in favor of hypothesis (B).

The last question is the extent to which the vertical transmission accounts for the memory of the intact animal. The present results show a remarkable stability of the memory: In the intact animal, where only a part of the anti-body-forming capacity is used in a normal secondary response, no decline at all would be seen for yearlong periods, when the starting level is supramaximal. A recent estimate by Makinodan and Peterson (12) gives a half-life of 26 weeks for the secondary antibody-forming potential against sheep red blood cells in the intact mouse. This figure agrees with our estimate of the memory decline.

SUMMARY

A calibrated cell transfer system allows detection of the anamnestic response to albumin without interference from the host's immune machinery; it was used to study the immunological memory of mouse spleen cell populations. The secondary antibody-forming capacity of the transferred cells was measured by challenging them at periods up to 6 months after transfer. The peak levels attained show a declining pattern in two phases: during the first month with a half-life of 15 days; thereafter, with a half-life of 100 days. The corresponding half-lives of the *cellular* memory are 26 and 190 days.

In the light of these and of radioinactivation data, immunological memory is defined as the persistence of a specifically determined stem cell line, along which the information necessary to give rise to an antibody-forming cell population is transmitted from mother to daughter cells.

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