Immunology, 1968, **14**, 503.

Immunogenicity of Human Serum Albumin: Decay in the Normal Mouse

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(*Received* 5*th May* 1967)

Summary. The disappearance of immunogenicity of human serum albumin (HSA) was measured by injection of 1 μg of this protein in normal mice and by transferring to them, at intervals, a fixed number of HSA-sensitized syngeneic spleen cells: subsequently the antigen binding capacity of the serum was determined. This system served to reveal the residual amount of immunogen present in the animal, since under these conditions the adoptive secondary response is proportional to the HSA available, and no antibody is formed by the host. The half-life of HSA immunogenicity was 17½ hours. A similar rate of decline (T = 16½ hours) was found by following ^{131}I coupled to the HSA in the circulation of the same mice. This demonstrates that the albumin antigen is not stored in an active form in non-pre-immunized mice.

INTRODUCTION

Antibody production can be looked upon as a result of specific cell differentiation initiated or influenced by the antigen. The role of the antigen—if any—in sustaining this cell differentiation for long time periods (immunological memory) is still not understood. To shed light on this point it is necessary to determine not only the presence of antigen molecules *in vivo* concomitant with the retained immune potential, but also whether they are in an operationally active state, i.e. to measure their immunogenicity.

Several authors (for review see Humphrey, 1960) have tested for the presence of antigen in organs of immune animals by transferring homogenates of organs to recipients capable of specifically reacting against the antigen under study. More recently Mitchison (1965) determined the persistence of stimulatory material for as long as 10 weeks by transferring primed lymphoid cells into mice which had previously received up to 100 mg of bovine serum albumin. In a preceding paper from this laboratory a method is described by which the presence of immunogenic bacterial lipopolysaccharide antigens and sheep red blood cell antigens could be demonstrated by transferring non-primed cells to lethally irradiated previously immunized hosts and subsequently measuring the primary antibody response produced by the transferred cells (Britton, Wepsic and Möller 1968).

In this paper we describe the measurement of the amount of immunogen present several days after the inoculation of microgram quantities of human serum albumin into mice, and the quantitative comparison of the decay of immunogenicity with the elimination rate from the circulation. We have taken advantage of a calibrated transfer system worked out to study immunological memory (Celada, 1966) in which the log antibody titre produced by a given number of transferred cells is proportional to the log dose of challenging antigen, over a 1000-fold range (Celada, 1967).

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The latter observation is in general agreement with the data of Perkins and Makinodan (1965) who studied the adoptive response to a particulate antigen. Mäkelä and Mitchison (1965) obtained a flatter and more irregular response with bovine albumin: this may be due to the very high cell doses transferred (the equivalent of 1 or 2 donors/recipient). The method we used for determining immunogenicity is similar to that of Mitchison (1965). A significant difference was to use a dose of antigen five orders of magnitude smaller, and consequently to follow its disappearance over a span of a few days instead of several weeks. This avoided the injection of immunogenic-paralysing amounts of antigen and allowed us to follow the rate of disappearance of the protein from the circulation in the same animals.

MATERIALS AND METHODS

Animals

All mice used were male CBA. At the time of transfer, donors were 4–5 months, and recipients 2 months old.

Antigen

Four times crystallized human serum albumin (HSA) batch 7749, Nutritional Biochemical Corporation, Cleveland, Ohio, was used for pre-immunization of mice serving as donors of the primed transfer cells (5 mg were injected subcutaneously 2 months before transfer in Freund's complete adjuvant, Difco laboratories, Detroit) and in some experiments for injections into recipients. In experiments where the elimination of HSA in the serum and the decay of immunogenicity were studied in parallel, ^{131}I-labelled HSA (The Radiochemical Centre, Amersham, England), was used. The counting of the radioactive serum samples was done at the same time, after the last sampling, in order to avoid the need to correct for the radioactive decay of ^{131}I.

Irradiation

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 and 0·5 mm Cu. The dose rate, at 50 cm from the source was 100 r/min. The dose delivered to prospective recipients was 500 r, given within 1 hour prior to cell transfer.

Spleen cell transfers

This operation was done as described by Celada (1966). Essentially a donor spleen cell suspension obtained by teasing the organ in Eagle's medium, filtrating it and adjusting the cell concentration to 5×10^7/ml, was injected intravenously into the irradiated recipients (0·2 ml/mouse). The latter were bled 10, 20 and 30 days (in some experiments 7, 14 and 21 days) after transfer. Each group of recipients consisted of eight to twelve mice.

Serology

Titration of anti-HSA antibodies in the serum of recipient mice was performed by the Farr Technique (Farr, 1958). For each test 0·25 ml of a dilution of the antiserum was mixed with the same volume of [^{131}I]HSA, at a concentration of 1 μg/ml. This reaction and the ammonium sulphate precipitation of antibody–antigen complexes were carried out at room temperature. The calculation of the antigen binding capacity of the antiserum was done from the 50 per cent binding of the [^{131}I]HSA present in the reaction

mixture, as described by Celada (1966). Both the calculation and the statistical treatment of the data were performed by computer.

For each bleeding the mean log titre was calculated. Peak log titres were utilized for calculating the decay of immunogenicity.

RESULTS

The design of a typical experiment is shown schematically in Fig. 1. As can be seen, several variables were studied at the same time. Group 1 was injected with 1 μg [^{131}I]HSA and was subject to calibrated bleedings at different times after administration of the labelled antigen. After all samplings were collected determination of presence of label in

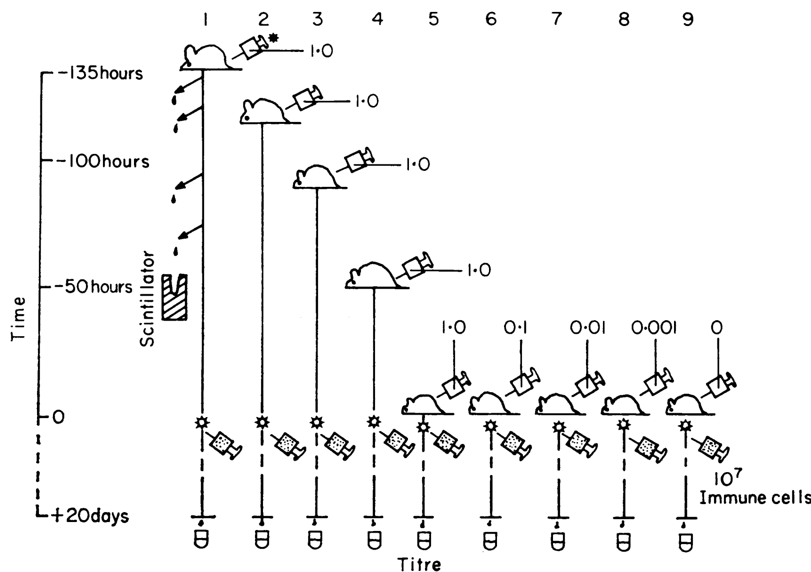

FIG. 1. Experimental design. The numbers on the top refer to nine groups of animals. Groups 1–8 each consist of eight to twelve mice. Group 9, twenty mice. The syringes represent i.p. injection of HSA solution whenever the dose (in μg) is indicated. Stippled syringes represent i.v. injection of a suspension of spleen cells from pre-immunized syngeneic donors. The open stars represent irradiation (500 r). The black star indicates ^{131}I-labelled HSA. The determination of anti-HSA titre is done by the Farr technique.

the serum was performed and the elimination of radioactive label in the serum could thus be calculated. Groups 2, 3 and 4 received the same amount (1 μg) of non-labelled HSA at indicated times before cell transfer. Groups 5–8 received different amounts of HSA (ranging from 1–0·001 μg) in order to establish the dose–response relationship. Group 9 did not receive any antigen and served as a control for the actual amount of antibodies produced by the transferred primed cells without further antigen stimulation.

At time 0 all groups were irradiated (500 r whole body irradiation) and re-populated with 10^7 pooled spleen cells obtained from five syngeneic animals pre-immunized with HSA in Freund's adjuvant. The bleedings of all tested animals were performed at 10, 20 and 30 days after cell transfer. All sera were titrated at the same time with the technique described above. In this experiment the peak titre was attained at day 20 after cell transfer.

The results obtained from Groups 5–8 show the amount of antibody formed to increase more than 100 times when the challenge increases from 0·0001 to 1 μg.

From the present data (Fig. 2) we could establish that the log titre is a linear function of the log of the challenge dose of antigen, within the range of 1–1000 ng.

In Fig. 2, the titres of a group (Group 3 in Fig. 1) of animals given 1000 g 90 hours before the cell transfer are also plotted. This is to illustrate the procedure used to determine the fraction of immunogenic HSA present in mice at a given time after administration of the antigen. By using the mean log ABC (in this case 1660 ng/ml) and the regression line

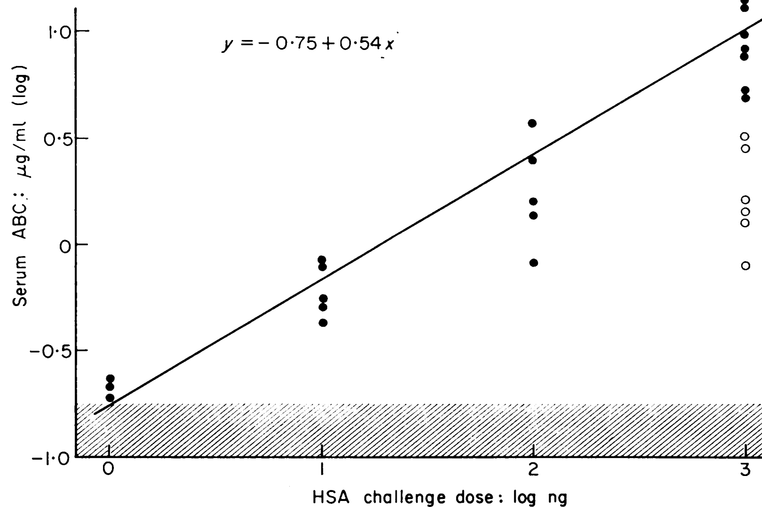

FIG. 2. Amount of antibody produced by 10^7 primed spleen cells as a function of the antigen challenge dose. Shaded area indicates the range of titres produced in the absence of challenge (= residual primary response). Ordinate: antibody titre expressed as serum antigen binding capacity (log μg HSA bound per ml of undiluted serum). Abscissa: HSA dose used as a challenge, expressed as log nanograms per mouse. Each symbol represents one mouse. ●, Injection of HSA at the time of cell transfer, ○, injection of HSA 90 hours prior to cell transfer.

TABLE 1

RESIDUAL IMMUNOGENICITY AS A PERCENTAGE OF THE ORIGINALLY ADMINISTERED DOSE OF ANTIGEN AT DIFFERENT TIMES AFTER INJECTION OF HSA IN NORMAL MICE

Experiment No.	Time (hours) after antigen administration												
	0	44	90	115	120	168	240	336	504	670	840	1020	1170
1	100					Traces	0	0	0	0	0	0	0
2	100				1·6		0	0					
3	100	25	4·5	1·3									

($y = -0{\cdot}75 + 0{\cdot}54x$) we can interpolate a value on the abscissa, 45 ng. This means that 4·5 per cent of the initial dose of 1000 ng is left in the animals at the time of cell transfer (in this case 90 hours after the antigen was initially administered).

Table 1 lists the decay pattern of immunogenicity of HSA in a series of three experiments. There was no evidence of presence of immunogenic antigen in the tests made with intervals between injection of 1 μg HSA and cell transfer ranging from 7–40 days, while titratable immunogenicity was found when the intervals were shorter. The slight discrepancy between experiments 2 and 3 (1·6 per cent residual immunogenicity at 120 hours versus 1·3 per cent at 115 hours) is statistically not significant.

In Fig. 3 we compare the disappearance rate of isotope labelled HSA from the circula-

tion with the decay of immunogenicity within the same experiment (Groups 1 and 2 from Fig. 1). The log counts per minute per millilitre blood and, respectively, the log ng of residual immunogenic HSA are plotted against the same time scale. The regression line of the disappearance of radioactivity was calculated from all samplings with the exclusion of the first one—2 hours after injection of the antigen—in order to allow a

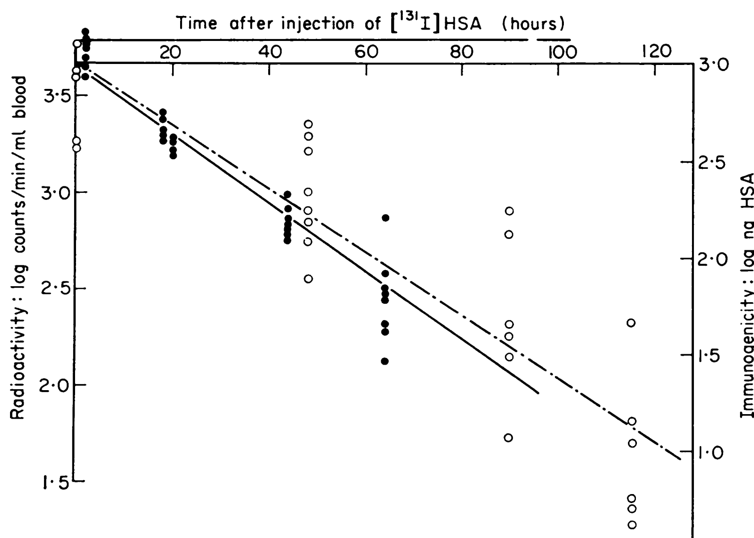

FIG. 3. Decline of ^{131}I in the serum (●) and of immunogenicity (○) following injection of 1 g HSA in normal mice. The half-life of radioactivity decline is 16·5 hours, that of immunogenicity is 17·5 hours.

satisfactory equilibration of the protein in the blood, extracellular and intracellular compartments (Garvey and Campbell, 1963). The slope constant was −0·19. When the amount of immunogenic HSA present was plotted against time a linear relation was found (correlation coefficient 0·94). The regression line of the decay of immunogenicity was calculated by using all points: time 0, −44, −99 and −115 hours; the resulting slope constant was −0·18. The figure shows the interpolation values of remaining immunogenic HSA calculated from the titre of each single recipient (for interpolation see Fig. 2). There is an apparent scatter of the individual values: the dispersion of titres (within groups the 95 per cent confidence limits of the mean log ABC are less than ±0·3, i.e. less than one dilution step) is doubled by the very operation of interpolation outlined before, which involves division of each value by the slope (0·5). For the sake of comparison of the two regression lines in Fig. 3, their extrapolations at time 0 have been graphically superimposed. The decay of label in the circulation and the decay of immunogenicity in the organism do not differ statistically from each other.

DISCUSSION

The data presented in this paper show: (a) that it is possible to determine quantitatively a foreign substance in an animal by utilizing—as a marker—its immunogenicity, and (b) that the antigen is not stored in an immunogenic form in non-immune animals but disappears from the organism at the same rate as from the circulation. The experi-

ments were done by transferring cells which produced a detectable secondary response whenever they encountered quantities of the antigen down to 0·001 μg. An essential feature of this system is the lack of detectable immunogenicity of less than 1 mg of soluble HSA when injected into non-primed mice.

Thus, we were able to quantify the residual antigen at various times, up to 115 hours after the injection of 1 μg HSA, by utilizing the proportionality of the log of antibody produced by the transferred cells to the log of the challenge dose.

The method of assessing the antigen by its immunogenicity was, in our hands, more sensitive than that of measuring radioactive label in the serum: while the latter allowed us to determine with certainty 100 ng of HSA, the former could detect 10. However, the sensitivity of the detection of radioactively labelled antigen could be substantially augmented by increasing the blood volume to be tested or, otherwise, by using [^{131}I]HSA with a higher specific activity although this might cause denaturation of the protein (Garvey and Campbell, 1963).

The decrease of immunogenicity with time was linear in a semi-log plot with a half-life of 17·5 hours. This is statistically not different from 16·5 hours which characterized the rate of disappearance from the circulation of the very same animals of a radioactive label attached to the antigen, an estimate in good agreement with the findings of other authors (e.g. Dietrich and Weigle, 1963). The coincidence of the two sets of results indicates that in the normal non-primed mouse given a small dose of antigen equilibrium is reached between the various body compartments and that HSA is not retained or concentrated in an immunogenic form where the transferred cells could reach it.

If we assume that the rate of catabolism is independent of the dose of antigen injected, then 100 mg of albumin should reach the stage of non-immunogenicity in about 2 weeks. This was not the case in the experiment of Mitchison (1965), where immunogenicity was found after 6–10 weeks. This may indicate that a drastic antigen treatment (probably at the same time immunogenic and paralysing) to the recipient, activates some different way of antigen retention or storage.

From the data obtained it is not possible to make any positive correlation between the persistence of immunogenic HSA and the duration of immunological memory to HSA in the mouse. On the contrary, the rapid half-life of immunogenic HSA and the very long half-life of the immunological memory to HSA may suggest that the immunogen plays no direct role in sustaining the specific immunological reactivity. However, it must be stressed that these determinations of the immunogenicity of HSA have been performed in unprimed mice whereas the memory studies by definition are performed in pre-immunized animals. It is possible that the antigen in the sensitized animal is stored in a physically (antibody precipitated?, particulate?) or topographically different way which would increase and prolong its immunogenicity. In fact, a different localization pattern of radioactively labelled antigens in primed and non-primed mice have been demonstrated by other authors (Nossal, Ada, Austin and Pye, 1965; McDevitt, Askonas, Humphrey, Schechter and Sela, 1966). To tackle this problem we are presently comparing the decay of immunogenicity in primed and non-primed mice, by using a modification of the present transfer technique.

ACKNOWLEDGMENTS

We thank Professor George Klein for generous support and Miss Cecilia Wijnbladh and Miss Rena Viefhaus for expert technical assistance.

This work was supported by Public Health Service Grant CA-3700 from the National Cancer Institute, Grant DRG-598 from the Damon Runyon Memorial Fund, by the Swedish Cancer Society, the Swedish Medical Research Council, the Magn. Bergwall Foundation and the Sir Samuel Scott of Yews Trust.

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