Immunology, 1964, 7, 449.

Immunological Unresponsiveness to Protein Antigens in Rabbits

I. THE DURATION OF UNRESPONSIVENESS FOLLOWING A SINGLE INJECTION AT BIRTH

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(*Received 4th October* 1963)

Summary. The immunological responses of rabbits to HSA, HGG or BSA were tested at various times later in animals which had received the corresponding antigens before or shortly after birth. As judged by the criterion of failure to show immune elimination of antigen, a high proportion of the rabbits remained unresponsive at times when it was calculated that all the originally administered antigen would have been eliminated from the circulation. Furthermore, removal of antigen by passively administered antibody failed to restore the capacity to respond. It is concluded that, in respect of the antigens used, their persistence in the extracellular body fluids is not a prerequisite for maintenance of immunological unresponsiveness.

Further administration of the same antigen to rabbits which had escaped from a state of specific immunological unresponsiveness generally produced a very weak response, and in a few instances resulted in a return to the unresponsive state.

When the cross-reacting antigens HSA and BSA were administered adsorbed on alum to rabbits made unresponsive by neonatal contact with BSA and HSA respectively, and at the same time a further dose of the original antigen was given, antibodies were formed which were specific for the second antigen and did not cross-react with the first. In only 1/9 animals was responsiveness to the first antigen restored. The significance of these results is discussed.

INTRODUCTION

Several authors have confirmed the original observations of Hanan and Oyama (1954) that administration of various soluble heterologous proteins to newborn rabbits results in a state of 'tolerance' or specific immunological unresponsiveness to the proteins administered (e.g. Dixon and Maurer, 1955; Cinader and Dubert, 1955). Their experiments were not, however, designed to test how long unresponsiveness would last after the first contact with the potential antigens. Smith and Bridges (1958) and Smith (1960), in a series of careful experiments, investigated the duration of unresponsiveness to bovine serum albumin (BSA) after first contact during the neonatal period with various amounts of this material. They concluded that maintenance of unresponsiveness required the persistence in the animal's circulation of antigen above a certain level (about 10^{12} molecules). This conclusion, if generally valid, would be important for understanding the mechanism of unresponsiveness. Experiments with BSA, human serum albumin (HSA)

and human γ-globulin (HGG), which were in progress at about the same time in this laboratory appeared to give qualitative support to Smith and Bridges's conclusions, and were quoted as doing so by Humphrey (1960). Continuation of these experiments showed, however, that immunological unresponsiveness to HSA and HGG persisted in a high proportion of animals considerably beyond the time when no more antigen could be expected to remain in the circulation. Furthermore, removal of persisting antigen from the circulation by means of heterologous antibody did not restore a state of responsiveness. During the course of these long-term observations, which are reported below, many of the animals eventually became able to give an immunological response to the antigens in question, but responses were often very poor and unlike those given by normal rabbits. Details of these findings and a possible explanation are discussed in an accompanying paper.

MATERIALS AND METHODS

Rabbits

Sandylops of either sex, bred at the National Institute for Medical Research, were mostly used, but a few albinos were also included. When adult they weighed 2·5–3 kg. During studies of elimination of ^{131}I-labelled proteins the rabbits were given drinking water containing 0·45 per cent NaCl and 0·005 per cent KI except in the case of pregnant does mentioned below.

Antigens

Crystallized bovine serum albumin (Armour) and crystallized human serum albumin ('reinst', Fabwerke-Hoechst) were used both for initial injections and subsequent testing. The human γ-globulin used for producing unresponsiveness was kindly given by Dr. W. d'A. Maycock (M.R.C. Blood Products Unit, Lister Institute, Elstree), whereas that used for subsequent tests was prepared from fresh human serum by chromatography on DEAE–cellulose (Peterson and Sober, 1956).

Antibodies

Rabbit anti-HGG and rat anti-HSA were prepared by administration of the antigens in Freund's complete adjuvant, followed by a course of intravenous injections of the corresponding antigen adsorbed on alum. The precipitable antibody contents of the final sera were: rabbit anti-HGG 5·8 mg./ml., and rat anti-HSA 3·2 mg./ml. γ-Globulin concentrates from each were prepared by chromatography on DEAE–cellulose, and iodinated as described below. By paper electrophoresis the preparations were shown to contain only γ-globulin.

Iodination

Iodination with ^{131}I was performed by the iodine monochloride method (McFarlane, 1958), at an average of 1 atom I per mol.

Production of Immunological Unresponsiveness

This was performed:

(a) *by neonatal injection* of a 5 or 10 per cent solution subcutaneously within 48 hours of birth, followed in some instances by a further injection 4 days later, or

(b) *by transplacental transfer.* The pregnant does received 450–700 mg. of antigen intravenously 8–10 days before parturition. The antigen was trace labelled with ^{131}I, in order to ascertain how much was present in the babies at birth and to check that immune elimination had not occurred from the mother. Plain drinking water was provided, without added iodide or chloride, since iodide not only becomes concentrated in the milk but is very poorly excreted by baby rabbits. One or more babies from each litter was bled from the heart, and the location of radioactive protein in the serum examined with a strip counter after electrophoresis on paper. Protein from the remainder of the serum was precipitated with 10 per cent trichloroacetic acid, and the amount of protein-bound radioactivity determined. From these measurements the amount of antigen transferred was calculated, on the assumption that the total was equal to the concentration per ml. serum $\times 10$ per cent of the body weight.

(c) *by transplacental transfer together with neonatal injection.* The method used was a combination of (b) and (a). The total amounts of antigen administered to the babies were 50–150 mg. of HSA, 6–60 mg. of HGG and 0·2–120 mg. of BSA.

Assessment of Immune Response

Ten to 20 mg. of ^{131}I-labelled antigen were injected intravenously, and the amount remaining in the body was followed by measuring whole body gamma radioactivity on alternate days for a total of 21–28 days in a whole body counter (Campbell, Cuthbertson, Matthews and McFarlane, 1956). In animals unresponsive to HSA or BSA the slope of the plots of log (antigen retained in the body) against time were constant; in those unresponsive to HGG there was a gradual slight decrease in the slopes, most marked during the first 7–10 days. In those which showed an immune response, an abrupt or gradual change of slope accompanied the formation of circulating antigen–antibody complexes and immune elimination (cf. Talmage, Dixon, Bukantz and Dammin, 1951; Dixon and Maurer, 1953). Most of the rabbits which were unresponsive to the first challenge, and some of those which showed immune elimination were re-challenged after various time intervals, either with a further quantity of ^{131}I-labelled antigen or with unlabelled antigen adsorbed on alum administered intravenously.

Measurement of Antibody

Precipitable antibody was measured by the quantitative precipitin technique (Kabat and Mayer, 1948), and antigen-binding capacity by the ammonium sulphate method of Farr (1958).

RESULTS

EVIDENCE FOR TRANSPLACENTAL TRANSFER OF ANTIGEN

After administration of 450–700 mg. of antigen to the mothers, the total amounts found in the babies (calculated as described above) were: HGG, 2–2·5 mg.; HSA, 0·1–0·3 mg.; BSA, 0·1–0·2 mg. Paper electrophoretic examination of the babies' sera showed that in those born from mothers given HGG or HSA, the radioactivity was confined to the γ-globulin and albumin regions respectively. In those born from mothers given BSA about two-thirds of the radioactivity was in albumin and one-third in the α-globulin region. This finding indicates that traces of α-globulin, known to be present in the BSA, were preferentially transferred to the foetus.

The amounts of antigen calculated as having crossed the placenta were used as a basis for further calculations of antigen persistence in the plasma. There was no evidence from these and other experiments not reported here that antigen transferred across the placenta was more effective in inducing prolonged unresponsiveness than was antigen injected shortly after birth.

CALCULATION OF ANTIGEN REMAINING IN THE CIRCULATION

In order to relate the persistence of immunological unresponsiveness to the amount of circulating antigen at the time of testing, it is necessary to use information available about the catabolism of the various antigens in rabbits at different ages. The catabolism of HSA

TABLE 1

HALF LIVES (DAYS) OF ^{131}I-LABELLED PROTEINS IN UNRESPONSIVE RABBITS

Age (months)	*HSA*	*HGG*	*BSA*
0–1½	6	12	6*
2	7	9*	7*
3	7	7*	7
4	7	part 5–6, part 7·5 } say 7	7
6	7	part 5–6, part 7·5 } say 7	7
8–10	7·5	part 5–6, part 7·5 } say 7	7
>10	8	part 5–6, part 7·5 } say 7	7

* Assumed. Other figures are the means of actual measurements.

and HGG in rabbits during the first 6 weeks of life has been measured by Humphrey (1961). Half lives at later stages of growth were obtained from measurements in unresponsive animals made in the course of the present experiments, and are listed in Table 1. The half lives of HSA and BSA are somewhat longer than those reported in rabbits by most other workers who have used similar proteins labelled with ^{131}I, although that for BSA is similar to the value reported by Smith (1960) using an immunochemical method of estimation. The explanation may lie in part in the fact that whole body radioactivity declines somewhat slower than plasma radioactivity (see, e.g., Campbell *et al.*, 1956), but may also reflect differences in the techniques of iodination and in the strains of rabbits used. In the case of HGG the slope of the elimination curve was not constant, but decreased gradually, indicating the presence of a fraction with a longer half life than that of the bulk of the material (cf. Cohen and Freeman, 1960). A compromise figure was therefore used, as indicated in Table 1.

From these figures can be calculated the fraction of initially injected antigen which remains in the body, on the assumption that the breakdown of trace labelled [^{131}I]proteins and of unlabelled proteins does not differ significantly. Strong evidence in favour of the truth of this assumption has been presented by McFarlane (1957) and by Freeman, Matthews, McFarlane, Bennhold and Kallee (1959). Table 2 contains the calculated figures which are used in subsequent sections of this paper, and which represent the best

assessment possible based on existing data. Except in the case of rabbits less than 3 months old the figures are based on extrapolation from whole body rather than plasma radioactivities, and might therefore be expected to take into account antigen retained intracellularly, provided that intracellular antigen does not lose its ^{131}I label or unless it forms an undetectably small fraction of the whole.

TABLE 2

FRACTION REMAINING OF ANTIGEN INJECTED AT BIRTH

Age (weeks)	*HSA**	*HGG**	*BSA**
4	$4{\cdot}5 \times 10^{-2}$	2×10^{-1}	$4{\cdot}5 \times 10^{-2}$
6	$1{\cdot}5 \times 10^{-2}$	$1{\cdot}1 \times 10^{-1}$	$1{\cdot}5 \times 10^{-2}$
8	$2{\cdot}1 \times 10^{-3}$	4×10^{-2}	$2{\cdot}1 \times 10^{-3}$
12	$1{\cdot}31 \times 10^{-4}$	$3{\cdot}72 \times 10^{-3}$	$1{\cdot}31 \times 10^{-4}$
16	$8{\cdot}2 \times 10^{-6}$	$2{\cdot}33 \times 10^{-4}$	$8{\cdot}2 \times 10^{-6}$
20	$5{\cdot}1 \times 10^{-7}$	$1{\cdot}46 \times 10^{-5}$	$5{\cdot}1 \times 10^{-7}$
24	$3{\cdot}2 \times 10^{-8}$	$9{\cdot}1 \times 10^{-7}$	$3{\cdot}2 \times 10^{-8}$
28	2×10^{-9}	$5{\cdot}7 \times 10^{-8}$	2×10^{-9}
32	$1{\cdot}25 \times 10^{-10}$	$3{\cdot}56 \times 10^{-9}$	$1{\cdot}25 \times 10^{-10}$
36	$9{\cdot}4 \times 10^{-12}$	$2{\cdot}22 \times 10^{-10}$	$7{\cdot}8 \times 10^{-12}$
40	$7{\cdot}05 \times 10^{-13}$	$1{\cdot}39 \times 10^{-11}$	$4{\cdot}9 \times 10^{-13}$
44	$6{\cdot}25 \times 10^{-14}$	$8{\cdot}7 \times 10^{-13}$	$3{\cdot}07 \times 10^{-14}$
48	$5{\cdot}55 \times 10^{-15}$	$5{\cdot}43 \times 10^{-14}$	$1{\cdot}92 \times 10^{-15}$
52	$4{\cdot}9 \times 10^{-16}$	$3{\cdot}4 \times 10^{-15}$	$1{\cdot}2 \times 10^{-16}$
56	$4{\cdot}35 \times 10^{-17}$	$2{\cdot}12 \times 10^{-16}$	$7{\cdot}5 \times 10^{-18}$
60	$3{\cdot}84 \times 10^{-18}$	$1{\cdot}33 \times 10^{-17}$	$4{\cdot}7 \times 10^{-19}$
64	$3{\cdot}4 \times 10^{-19}$	$8{\cdot}3 \times 10^{-19}$	$2{\cdot}94 \times 10^{-20}$
68	3×10^{-20}	$5{\cdot}2 \times 10^{-20}$	$1{\cdot}84 \times 10^{-21}$
72	$2{\cdot}66 \times 10^{-21}$	$3{\cdot}25 \times 10^{-21}$	$1{\cdot}15 \times 10^{-22}$
76	$2{\cdot}35 \times 10^{-22}$	$2{\cdot}03 \times 10^{-22}$	$7{\cdot}2 \times 10^{-24}$
80	$2{\cdot}08 \times 10^{-23}$	$1{\cdot}26 \times 10^{-23}$	$4{\cdot}5 \times 10^{-25}$
84	$1{\cdot}84 \times 10^{-24}$	$7{\cdot}9 \times 10^{-25}$	$2{\cdot}82 \times 10^{-26}$
88	$1{\cdot}63 \times 10^{-25}$	$4{\cdot}95 \times 10^{-26}$	$1{\cdot}77 \times 10^{-27}$
92	$1{\cdot}44 \times 10^{-26}$	$3{\cdot}1 \times 10^{-27}$	$1{\cdot}11 \times 10^{-28}$

* 1 mg. of HSA or BSA $\simeq 8{\cdot}85 \times 10^{15}$ molecules.
1 mg. of HGG $\simeq 3{\cdot}75 \times 10^{15}$ molecules.

RELATIONSHIP BETWEEN RETAINED ANTIGEN AND IMMUNOLOGICAL UNRESPONSIVENESS

Rabbits which had received antigen around the time of birth were re-injected intravenously with the same antigen labelled with ^{131}I after periods ranging from 9 to 112 weeks, and its elimination was followed for at least 3 and usually 4 weeks. This is a sufficiently long period of time to be certain that even a weak primary response in a normal animal would be observed. The amounts of antigen used for testing were 10–25 mg. in all but a few instances, when 50 or 100 mg. were used. Since 28 days of observation represents about four half lives, the antigen remaining in the circulation at the end of this time, following an initial injection of 20 mg., would be 1·25 mg. (equivalent to about 0·005 mg./ml. in the plasma).

Most rabbits were retested with the same antigen after further intervals of time, sometimes more than 1 year. Throughout the period between first and subsequent injections the rabbits all remained in good health.

Since the purpose of this communication is to relate the presence or absence of unresponsiveness to the amount of antigen remaining at the time of testing, the findings are given in terms of the number of molecules present in the body (calculated from Table 2) rather than of the number of weeks since the last injection.

The results with each of the antigens, on first testing, are shown in Fig. 1. Even though the number of animals in each group is not large (twenty-nine with HSA, twenty-three with HGG and sixteen with BSA) it is evident that a considerable proportion in each group (8/14, 11/15 and 2/5 respectively) had remained unresponsive at times when the estimated number of residual molecules of intact antigen was less than 10,000, and even less than one.

The findings recorded in the columns representing the presence of <1 molecule of antigen include those for some rabbits which had remained untested for many weeks since this level of antigen was calculated to have been reached. It was among such rabbits that

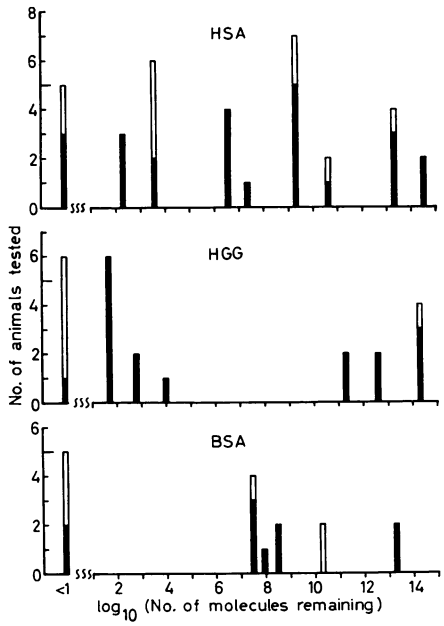

FIG. 1. Relationship between number of molecules of HSA, HGG or BSA, estimated to remain in rabbits (after injection at birth) and the presence or absence of immunological unresponsiveness to a first subsequent injection. Black columns—rabbits not responding; open columns—rabbits showing immune elimination. The left hand columns (abscissa <1) include animals from which it is calculated that antigen had disappeared up to 6 months previously.

occurred most of those which were found to be responsive. An interesting feature which emerges from Fig. 1 is the unpredictable nature of the results for any given animal, since certain of the rabbits showed immune elimination of the antigen when tested at a time when as many as 10^{13} or 10^{14} molecules remained in the circulation.

Most of the rabbits found to be unresponsive were tested again later without any intervening treatment, and the findings are shown in Fig. 2. They are qualitatively similar to those in Fig. 1, though the proportion of animals unresponsive at all stages is substantially higher. This may be explained by the fact that each group had already been selected for unresponsiveness by the first test, but more probably by the fact that these animals were by then older. It has been shown that with increasing age the amounts or frequency of administration of antigen required to maintain unresponsiveness decline (e.g. Mitchison, 1962).

THE EFFECT OF ELIMINATING ANTIGEN BY ADMINISTRATION OF ANTIBODY

Two rabbits which had received 40 mg. of HSA at birth, and at 12 weeks were estimated to contain 5 μg. of the antigen, were injected intravenously with 8 mg. of ^{131}I-labelled rat γ-globulin containing 1·2 mg. of precipitating anti-HSA. Two other rabbits which had received 30 mg. of HGG at birth, and still contained about 0·11 mg., received 12 mg. of ^{131}I-labelled rabbit γ-globulin containing 3 mg. of anti-HGG. The radioactivity remaining in the rabbits was measured daily. After 8 days all the rat γ-globulin had been eliminated (by immune mechanisms), and after 10 days the injected rabbit γ-globulin had fallen to

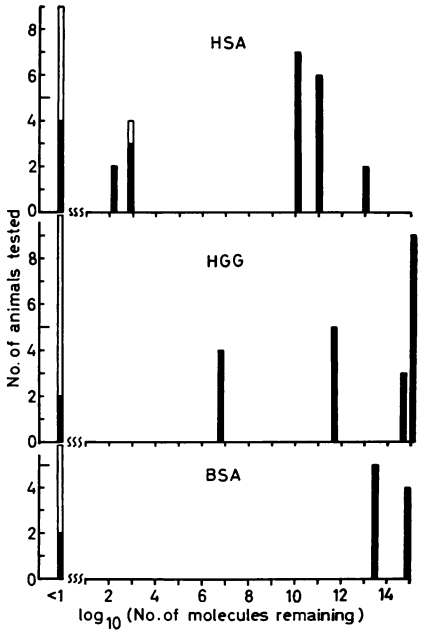

FIG. 2. Relationship between number of molecules of HSA, HGG or BSA remaining and state of immunological responsiveness following second or third administration of the antigens to rabbits previously found to be unresponsive. Black columns—rabbits not responding; open columns—rabbits showing immune elimination. The left hand columns (abscissa <1) include animals from which it is calculated that antigen had disappeared up to 6 months previously.

33–40 per cent of the original level, it then being assumed that any of the residual neonatally injected antigens would by now have been eliminated. Further 25 mg. quantities of the original antigens, labelled with ^{131}I, were now administered intravenously, and their elimination was followed for 24 days. No evidence of immune elimination was obtained, and when retested 33 weeks later all the rabbits were still unresponsive to the original antigens.

THE EFFECT OF FURTHER IMMUNIZATION WITH THE SAME ANTIGEN ON RABBITS WHICH HAD ESCAPED FROM UNRESPONSIVENESS

A number of rabbits which had at one time been unresponsive to BSA or HGG, but had shown (delayed) immune elimination of the antigen on subsequent re-testing, were later challenged again with the same antigen. The response was measured either in terms of the pattern of antigen elimination or of the antigen-binding power of the serum, or both.

Somewhat surprisingly some of the rabbits were found to be once more unresponsive by these tests. The findings are given in Table 3.

Even the rabbits which made antibody produced very little compared to previously untreated rabbits which received similar amounts of antigen. Some findings relating to this are the subject of an accompanying paper.

TABLE 3

EVIDENCE FOR OCCASIONAL REVERSION TO UNRESPONSIVENESS

Antigen	*Rabbit No.*	*Test dose resulting in first (delayed) immune elimination (mg.)*	*Subsequent treatment*	*Result*
HGG	93/58, 94/58 110/58, 111/58 112/58	15	15 mg. i.v. 21 months later	Immune elimination
	121/58	25	10 mg. i.v. 1 month later	Immune elimination
	14/59	25	25 mg. i.v. 7 months later	Unresponsive
BSA	58/58, 60/58 81/58, 96/58	10	i.v. course of alum precipitated BSA (total 18 mg.) 7 months later	Immune elimination and detectable antibody
	59/58	10	i.v. course of alum precipitated BSA (total 18 mg.) 7 months later	Probably unresponsive; antigen binding $<0{\cdot}002$ mg./ml.
	95/58	10	25 mg. i.v. 9 weeks later	Unresponsive
	99/58	10	25 mg. i.v. 3 months later	Unresponsive
	100/58	10	25 mg. i.v. 3 months later	Unresponsive

SPECIFICITY OF UNRESPONSIVENESS

Although the fact is well attested (cf. review by Smith, 1961) that immunological unresponsiveness following neonatal contact with antigen is highly specific, the fact that rabbits were available which were unresponsive to two cross-reacting antigens, BSA and HSA, offered an opportunity to test the specificity under stringent conditions. Thus in sera from normal rabbits immunized by repeated injections of HSA some 15 per cent of the anti-HSA is precipitable by BSA (e.g. Weigle, 1961a), and *vice versa* (author's observation). If the state of unresponsiveness applied to all the antigenic determinants of the one antigen, immunization with the other might be expected to lead to antibody formation only against those determinants which were not common to both. The experiments described below were carried out before the publication by Weigle (1961b) of his finding that intravenous injection of cross-reacting antigens can restore responsiveness to an antigen to which rabbits have been made unresponsive. They are reported because the results, though limited to a few animals, are at first sight not in accord with his findings.

Four rabbits which had proved unresponsive to HSA 6 months previously and five which had proved unresponsive to BSA 1 month previously each received 10 mg. of the corresponding antigen labelled with ^{131}I, to test whether they remained unresponsive, and at the same time a course of intravenous injections was begun of the unlabelled cross-reacting antigen precipitated with alum. The courses consisted of a total of 18 mg. of

antigen, administered in six injections over a period of 13 days. Six days later the rabbits were bled, and the amounts of precipitating antibody in their sera were estimated. These were as follows: anti-BSA in rabbits unresponsive to HSA, mean 1·65 mg./ml., range 1·1–2·0 mg./ml.; anti-HSA in rabbits unresponsive to BSA, mean 0·73 mg./ml., range 0·12–1·7 mg./ml. The antibodies were in each case specific for the antigen which elicited them, and showed no evidence of cross-reaction. During and subsequent to the administration of the cross-reacting antigens 4/4 of the rabbits previously unresponsive to HSA and 4/5 of those unresponsive to BSA failed to show any increased rate of elimination of the labelled protein. They were still unresponsive when re-tested 1–2 months later.

The rabbits unresponsive to HSA and immunized against BSA were given a further similar course of injections of alum precipitated BSA (without HSA on this occasion) 6 months after the first, and gave typical secondary responses: mean 7·7 mg. antibody/ml., range 3·8–9·4 mg./ml. Again there was no cross-reaction with HSA. However, when the same rabbits were challenged a year later with 15 mg. of [^{131}I]HSA intravenously, 2/4 showed typical primary and 2/4 markedly delayed immune elimination. Since the time interval before this last test was long it is uncertain whether or not the return of responsiveness was attributable to the two courses of BSA given 12 and 13 months earlier.

DISCUSSION

The results presented above indicate that following contact with HSA, HGG or BSA in the perinatal period rabbits may remain unresponsive to these antigens without further contact for very long periods of time. According to calculations based on extrapolation from clearance rates at different ages of proteins trace labelled with ^{131}I, unresponsiveness persisted in about half the rabbits tested at a time when few or no molecules of antigen remained in the circulation or in the body. The validity of this conclusion depends upon how far such extrapolation is justified, as well as upon the correctness of the assumed clearance rates. The latter are probably reasonably correct in respect of HSA or BSA, the slopes of whose elimination curves were rather constant, but it is possible in the case of HGG that small amounts of material with a half life in excess of 7 days could have produced a sufficient cumulative error to cause significant underestimation of the amount remaining after many months. Clearance rates of ^{131}I-labelled proteins from the body are certainly well correlated with their disappearance from the extracellular fluids, and this in turn is well correlated with the recovery of iodide or mono- and di-iodotyrosine in the urine. The dehalogenases which split off iodine from iodotyrosines or thyroxine are generally assumed not to act on intact iodinated proteins but only after these have been broken down intracellularly to small peptides; in the case of the powerful dehalogenases of muscle and liver this has been shown by Tata (1960) to be so. Nevertheless there is no proof that amounts of labelled antigen too small to affect the elimination curves may not persist intracellularly. Haurowitz, Reller and Walter (1955) and Hawkins and Haurowitz (1959) have reported the presence of labelled antigens in liver and spleen after intravenous injection, and Garvey and Campbell (1956) have demonstrated that antigenically effective haemocyanin may persist in the liver of rabbits for many weeks, despite the fact that its half life in the circulation in this species is only about 12 hours (Humphrey, unpublished). It is wise to assume, therefore, until direct evidence is available, that minute amounts of antigens may persist within cells, including those involved in the immune response, even when the vast majority has been eliminated.

A further question to be considered is the adequacy of immune elimination following intravenous injection of 10–25 mg. of the antigen as a criterion of responsiveness. Provided that elimination is followed for three or four half lives, and that the normal half life is a week or more, this method provides a sensitive indication of the time of onset and to some extent of the intensity of the immune response. Normal adult rabbits from our stock show immune elimination following first intravenous injections of 2–100 mg. of the antigens employed in the native state. Occasional adult rabbits have not responded to first injections of 1 mg. of HGG, but showed immune elimination on subsequent reinjection of the same material. Insufficient numbers of normal rabbits have been tested by intravenous injections of the antigens, native or adsorbed on aluminium hydroxide, to allow a definite statement that non-responders are never found, but over the past 10 years out of at least twenty rabbits injected with 12 mg. of each of the antigens in Freund's complete adjuvant every one has responded. Bussard (1962) however has criticized the validity of absence of immune elimination of [^{131}I]HSA as a criterion of unresponsiveness on the grounds that some non-precipitating antigen–antibody complexes containing HSA may be detected in the serum by precipitation with 33 per cent saturated ammonium sulphate at a time when the slope of the elimination curve shows very little increase. This possibility was not explored systematically in the experiments described above, but antigen binding capacity of three sera from rabbits apparently unresponsive to HSA and of four from rabbits apparently unresponsive to BSA was tested by the Farr technique in the presence of *added* ^{131}I-labelled antigens, 4 weeks after the initial injection of ^{131}I-labelled antigen. In none was significant binding detected, although when slow and delayed immune elimination had occurred antigen binding was quite definite (see Humphrey, accompanying paper). Furthermore no antibodies were detectable in these sera by the tanned cell agglutination method. In fact Bussard's findings are in general agreement with those presented above, in that he tested rabbits 2 years after neonatal administration of 0·8–10 mg. of HSA and 2/8 rabbits showed no break in the elimination curve following i.v. administration of 20 mg. of labelled HSA and 1/8 showed an indefinite break. When the rabbits were retested 4 months later 2/6 showed no break in the curve and in their sera no soluble complexes were detected.

These results appear to indicate that extracellular persistence of the antigens studied is not a prerequisite for maintenance of immunological unresponsiveness, and this conclusion is reinforced by the evidence presented that passive administration of excess antibody, which in the case of rat anti-HSA was shown to have undergone immune elimination in its turn, did not abrogate the state of unresponsiveness. The conflict between these findings and the tentative conclusion of Smith and Bridges (1958) that unresponsiveness to BSA required the persistence of some 10^{12} molecules in the circulation is more apparent than real, insofar as these authors were concerned with the level above which *all* their rabbits were unresponsive. Even above this level a few of the rabbits in our experiments were capable of responding to this antigen. Mitchison (1962) studied the return of responsiveness in chickens made unresponsive to allogeneic fowl or turkey erythrocytes, after these had been eliminated from the circulation by passively administered antibody. In the case of turkey erythrocytes responsiveness returned after an interval of not more than 5 days, but in the case of allogeneic fowl erythrocytes a longer interval (up to 215 days) was required. Since it is now generally agreed that neonatally induced immunological unresponsiveness and immune paralysis induced in adult animals are essentially similar, the paralysis of adult mice by pneumococcal polysaccharides may be included for com-

parison. These polysaccharides are very rapidly removed from the blood stream by cells of the reticulo-endothelial system, and yet specific immune paralysis may persist for a lifetime. As has been shown by Felton, Prescott, Kauffman and Ottinger (1955), in this case the antigen persists intracellularly for many months in a detectable form. Thus induced unresponsiveness to proteins, erythrocytes and polysaccharides all appear to fall into line, in that the continued presence of extracellular antigen is not an absolute prerequisite for the persistence of the unresponsive state, at least for a certain length of time.

Inspection of Figs. 1 and 2 does not suggest any factor, other than time or chance, as determining whether or not a given animal would be responsive at any given level of residual antigen, although there is a suggestion in Fig. 2 that unresponsiveness was more likely to persist at lower levels of antigen in rabbits which had already been proved unresponsive after the first administration. Since Mitchison (1962) showed in chickens that unresponsiveness to allogeneic erythrocytes persisted in the absence of the antigen for longer the older the animals were, it seems likely that the greater age of the rabbits in the experiments of Fig. 2 compared with those of Fig. 1 is sufficient to account for the difference.

In discussing theories to account for the acquisition of tolerance, and the eventual loss of tolerance in the absence of antigen (after a period of time which depends upon the antigen and the age of the animal), Mitchison (1962) argued that most findings could be explained by assuming that immunological paralysis or tolerance depends mainly on some block caused by antigen in the maturation of those stem-cells which would normally give rise to cells capable of elaborating antibody in response to the antigen in question. Cellular maturation (whose rate would diminish with increasing age) on the part of the hitherto blocked stem-cells occupies the interval between loss of antigen and the gain of reactivity. Return of responsiveness would thus depend on recruitment of a population of responsive cells from precursors which had lost their block to maturation, either by gradual loss of a specific inhibitor—perhaps intracellular antigen—or by its dilution in the course of cell proliferation. If this argument is accepted, it might even be pushed back one stage further, by postulating that the stem-cells, which can be paralysed by antigen, are themselves derived from precursors which are functionally so undifferentiated that they are as yet not recognizably precursors of immunologically competent cells, and consequently insusceptible to paralysis. No assumptions about the unblocking of the blocked cells would then be required. The rate of return of responsiveness would then depend upon the rate at which the undifferentiated precursors gave rise to the stem-cells from which immunologically competent cells derived. This is however only speculation. The results do not permit distinction between these possibilities, nor were sufficient animals followed for long enough to establish whether a rate of return of immunological competence could be calculated.

The apparent reversion of a few rabbits to a state of unresponsiveness on further treatment with the same antigen is of considerable interest. A similar unexpected finding is mentioned in the protocols of an experiment in rabbits by Denhardt and Owen (1960), and a more systematic study of the phenomenon has recently been reported by Hasek and Ruza (1962) who used ducks made unresponsive to allogeneic erythrocytes first administered shortly after hatching. Evidence is presented in an accompanying paper (Humphrey, 1964) that when the capacity to produce antibody returns the amounts of antibody made are unexpectedly small, and—in some animals at least—this antibody is directed against only a restricted part of the total antigen mosaic. It seems possible that there is a stage during the return of responsiveness in which the total number of cells able to respond to

any aspect of *the antigen in question* is very small, and that in respect of this antigen only the adult animal resembles a newborn animal, or one treated by metabolic inhibitors or radiomimetic drugs. In these circumstances an amount of antigen which would normally stimulate an immune response is sufficient to cause long lasting immunological paralysis (see, e.g., Humphrey, 1963).

The fact that administration of alum precipitated HSA or BSA to rabbits unresponsive to BSA and HSA respectively apparently ended the unresponsive state in only 1/9 animals is surprising in view of Weigle's (1961b) clear-cut evidence that these cross-reacting antigens were able to restore the immune response to each other in a high proportion of unresponsive rabbits. However the conditions of the experiments described above differed from those of Weigle in two respects: the cross-reacting antigen was adsorbed on alum, and 10 mg. of the original antigen was administered at the same time. The latter may somehow have re-inforced the unresponsive state, although in our present state of ignorance concerning both the origin and the termination of unresponsiveness it is difficult even to speculate how this might have occurred. Nevertheless these findings may suggest an important limitation to attempts to explain autoimmune phenomena in terms of Weigle's observations.

ACKNOWLEDGMENTS

I am most grateful to Dr. N. A. Mitchison for discussing the interpretation of the findings, and to Mrs. Rosemary de Rossi for her able technical assistance.

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