Tolerance of Erythrocytes in Poultry: Loss and Abolition

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Summary. Fowls which tolerate foreign erythrocytes, but which have not received a transplant of viable cells, eventually lose tolerance when the supply of erythrocytes is discontinued. The loss follows, at an interval, after the foreign erythrocytes have been eliminated by the normal mechanism for removal of aged cells; earlier loss can be brought about by eliminating the foreign cells with passive antibody. The interval required for loss varies, for tolerance of homologous erythroctyes, from less than 5 to over 215 days. The age of the tolerance bird, and hence the duration of exposure to antigen, is the main factor influencing the interval required; the preceding level of tolerance exerts comparatively little effect. An attempt to terminate tolerance by adoptive immunization was only partially successful.

INTRODUCTION

Tolerance of erythrocytes, of the type described in the preceding paper, is here used to investigate the relationship between the presence of antigen and the maintenance of tolerance. Erythrocytes have a limited life-span, which ensures that antigen disappears from the circulation after a course of transfusion has been stopped. Foreign erythrocytes may also be removed from the circulation by passive immunization. By means of these methods the maintenance of tolerance has been tested after intervals devoid of circulating antigen. The precise time of disappearance of antigen was ascertained by labelling the transfused cells with ^{51}Cr and transfusion of labelled blood was also used to test the condition of tolerance. The fowls which were subjected to these tests varied in age and degree of tolerance, so that the influence of these factors on the stability of tolerance could be detected. For the purpose of comparison, an attempt was made to abolish tolerance by adoptive immunization.

MATERIALS AND METHODS

Materials and methods were used as described in the preceding paper(Mitchison, 1962): White Leghorn fowls or turkeys as blood donors, Brown Leghorns as recipients, and ^{51}Cr for labelling erythrocytes. As before, the blood received 10,000 r. before transfusion, in order to prevent induction of long-lasting tolerance by leucocytes.

RESULTS

LOSS OF TOLERANCE AFTER NON-RENEWAL OF ANTIGEN

Chickens were first rendered tolerant of homologous or heterologous erythrocytes; while under treatment they were handled in groups. Each group hatched from a single setting of eggs, and subsequently, up to the time of test, received uniform treatment with

blood from a single donor. The treatment consisted of a series of transfusion given in quantities and at intervals according to the standard schedule described in the preceding paper. Several birds lost tolerance spontaneously under treatment, and were discarded when their half-elimination time ($T_{\frac{1}{2}}$) of ^{51}Cr radioactivity fell below 3 days. Tests were then carried out on the tolerant birds, according to the design illustrated in Fig. 1. After
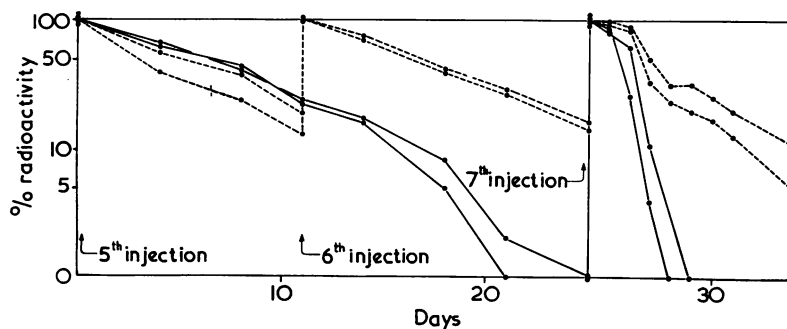

FIG. 1. Loss of tolerance after discontinuing transfusions. Four chickens tolerant of their fifth injection of turkey erythrocytes; two only (broken line) receive the sixth injection, and hence remain tolerant.

the last tolerance-maintaining transfusion each group was split up into sub-groups. One of the sub-groups usually served as a control, which received another transfusion with little or no delay after the radioactivity from the preceding transfusion had been elimini-ated. The remaining sub-groups were transfused after various times of delay, so that they had to pass an interval devoid of circulating antigen. The time of commencement of the interval was calculated, as in the preceding paper, by linear extrapolation from the radio-activity of the last two samples of blood, discarding samples in which the activity fell below 5 per cent of the initial value.

The effect produced by the intervals devoid of antigen are shown in Tables 1, 2 and 3. In most cases the state of tolerance either remained unaltered, as judged by the rate of elimination of the next transfusion, or else was definitely lost. Tolerance is assumed to have been retained in those birds in which the value of $T_{\frac{1}{2}}$ remained within the normal limits of variation, as set by the preceding transfusion. Equally, immune elimination — accelerated elimination after an initial lag-phase — was taken to indicate the loss of tolerance. A less clear-cut result was obtained in a few cases, in which the value of $T_{\frac{1}{2}}$ fell appreciably but without signs of immune elimination. Most of the birds in this doubtful category were retransfused after their challenge transfusion, without delay. When this second transfusion gave a value for $T_{\frac{1}{2}}$ of less than 3 days, tolerance is assumed to have been lost; but more commonly the second transfusion was eliminated at a slower rate than the first. This apparent recovery of tolerance may reflect a genuine reduction in the rate of production of antibody, but can probably be accounted for in the following way. Few, if any, of the birds which entered the test did so in a condition of complete tolerance: most of them were eliminating the foreign erythrocytes at a rate slightly higher than their own, and the foreign cells must presumably have been absorbing some antibody. This antibody was free to accumulate during the interval devoid of antigen, and could reach a higher concentration to confront the first batch of transfused cells. As expected on this hypothesis, the recovery was found most frequently among the birds which entered the test in a poor state of tolerance.

The groups of fowls are arranged in Tables 1–3 according to their age at the time of test. Within each age-group, a relationship can immediately be seen between the length of interval and the maintenance of tolerance: the longer the interval devoid of circulating antigen, the more likely becomes the loss of tolerance. The short intervals allowed in the control sub-groups were followed only occasionally by loss, which was then equivalent to

TABLE 1

THE EFFECT ON YOUNG FOWLS OF DISCONTINUING TRANSFUSIONS OF HOMOLOGOUS BLOOD

Days of age at:		$T_{\frac{1}{2}}$ at last maintenance transfusion — interval without ^{51}Cr in blood — $T_{\frac{1}{2}}$ at challenge transfusion (days)
Last maintenance transfusion	Challenge transfusion	
13	28	4.3–0–5.7; 4.0–0–6.2; 3.8–0–4.9; 3.7–0–5.6; 3.2–0–6.3; 3.0–0–6.5
	67	*4.3–36–ie3*; 3.9–37–ie3; 3.6–37–ie3; 3.5–38–ie3; 3.5–38–ie3; 3.4–39–ie3*
13	34	5.8–4–5.5; 6.3–5–3.6; *4.7–5–ie3; 4.3–9–ie3*
	39	*6.1–8–2.5; 5.5–9–2.4; 5.3–9–ie3, 4.8–10–ie3; 4.2–11–ie3*
	44	*5.8–14–ie2; 5.1–14–ie2; 4.5–14–ie2; 4.5–14–ie2; 3.9–15–ie2; 3.8–15–ie2*
23	29	3.6–0–5.5; 3.5–0–5.5
	51	5.8—12–2.3; 3.7**; *3.2–14–ie4, ie3;* 3.0–15–2.8, 3.0
	70	*4.9–30–ie4*
28	49	6.3–0–6.1; 6.2–0–6.2
	59	6.7–8–5.6; *6.1–11–ie4*
	67	*6.5–18–ie3; 4.9–18–ie4*
	87	*5.7–37–ie3; 5.6–38–ie3*
29	49	5.1–1–5.1; 4.8–4–1.9, 3.8; 4.6–2–4.7, 3.2; 4.1–3–3.2
	56	4.5–9–3.2, 4.7; *4.5–10–ie3;* 4.3–7–3.8; *3.5–11–2.8, ie2*
	63	4.9–16–3.9; *4.5–16–ie2; 3.5–18–ie2;* 4.2–19–3.4; *3.1–19–ie3*
31	51	5.6–2–6.0; 4.9–6–4.2; 4.0–6–3.5; 3.2–6–3.0
	55	*5.7–6–ie3;* 5.5–6–4.0; *5.3–8–ie3;* 5.2–6–4.4
	58	5.7–9–3.6; 4.6–9–3.6; 4.2–14–3.5

Italicized results are taken to indicate loss of tolerance.
* ie3 denotes immune elimination on third day after transfusion.
** 2.3, 3.7 denote values of $T_{\frac{1}{2}}$ at two successive transfusions after the gap.
The same conventions apply to Tables 2–5.

the 'spontaneous' loss shown in Fig. 3 of the preceding paper. Among the younger fowls (Table 1) an interval could always be found of sufficient length to ensure loss of tolerance; this did not hold for the older fowls (Table 2), although longer intervals evidently increased the chances of loss. The form of the relationship can be seen more clearly by arbitrarily grouping the data of Tables 1, 2 and 4, and accumulating according to the

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<div align="center">TABLE 2</div>

<div align="center">THE EFFECT ON OLD FOWLS OF DISCONTINUING TRANSFUSIONS OF HOMOLOGOUS BLOOD</div>

Days of age at:		$T_{\frac{1}{2}}$ at last maintenance transfusion — interval without ^{51}Cr in blood — $T_{\frac{1}{2}}$ at challenge transfusion (days)
Last maintenance transfusion	Challenge transfusion	
59	79	6.2–0–5.5
	115	*5.6–36–ie3*
70	92	8.4–0–7.4; 5.6–3–6.5
	129	*3.7–41–ie3; 3.7–41–ie3; 3.0–43–ie3*
123	153	8.8–5–4.6; 8.5–8–3.9; *7.0–9–ie4*; *5.5–10–ie4*
	163	8.4–19–6.1; 8.1–18–8.4; 7.4–19–3.3, 6.1; 4.9–23–4.5
131	152	5.8–1–4.9; 5.5–3–4.1; 4.9–4–5.0
	182	8.4–28–7.0; 7.4–29–2.5, 3.9; 6.7–33–4.5, 5.8; 4.4–34–3.8, 6.2
152	182	5.0–8–7.2; 4.9–10–3.4, 8.0; 4.1–12–5.8
173	233	4.6–36–8.1
	246	8.4–46–11.0; 6.1–51–5.7; 6.1–52–3.4, 6.0; 4.5–53–2.3, 7.6
182	202	4.5–2–5.8; 3.8–5–6.2
	232	7.2–30–5.0, 7.3; 7.0–30–9.4
202	261	8.0–35–8.0
241	311	9.0–44–6.5; 8.0–44–6.2; 6.5–45–5.2; 5.5–44–3.6, 3.5
250	317	7.3–40–5.1
	485	11.0–213–8.5
311	543	*6.5–210–ie4; 6.2–210–ie4*; 5.2–213–4.8, 5.6; 3.5–215–3.3, 4.7

<div align="center">TABLE 3</div>

<div align="center">THE EFFECT OF DISCONTINUING TRANSFUSIONS OF TURKEY BLOOD</div>

Days of age at:		$T_{\frac{1}{2}}$ at last maintenance transfusion — interval without ^{51}Cr in blood — $T_{\frac{1}{2}}$ at challenge transfusion (days)
Last maintenance transfusion	Challenge transfusion	
40	53	4.9–0–5.2; 4.5–0–3.0; 4.3–0–4.8; 3.5–0–5.6
	67	*5.8–5–ie3; 5.5–4–ie3; 4.9–7–ie3; 4.1–8–ie4*

method of Reed and Muench (1938), as shown in Fig. 2. The time needed for the loss of tolerance appears to increase with age; in other words, tolerance becomes progressively more stable with age and increased period of exposure to antigen. By 1 year of age, more than half the chickens tested could remain tolerant for a further period of 7 months without circulating antigen.
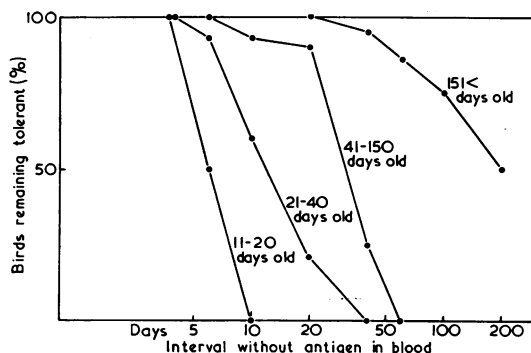

FIG. 2. The effect of age on the length of interval needed to abolish tolerance.

The pre-existing level of tolerance appears to exert comparatively little influence on the outcome of these tests. Within each sub-group, birds which entered the test with a high value for $T_{\frac{1}{2}}$ tended to retain tolerance most often. But only a minor stabilizing effect can be detected, after due allowance has been made for the shorter interval devoid of antigen which these birds passed through. Occasionally the reverse is observed, when a state of tolerance which seems weakest to begin with endures for longest.

Chickens which lost tolerance under test did not thereby become actively immune. Any automatic acquisition of immunity should be detectable, for immunized birds eliminate erythrocytes earlier than untreated birds (cf. Table 2 in the preceding paper). Following the loss of tolerance, immune elimination took place most frequently on the third day after transfusion, which is the time usual among untreated birds in this combination of donor and recipient. Immune elimination did take place before the third day slightly more frequently than would have been expected among untreated birds, but this can be accounted for by the accumulation of antibody from partial tolerance that has already been postulated. Belated immune elimination occurred as well, presumably when reactivity had not returned in full.

LOSS OF TOLERANCE AFTER PASSIVE IMMUNIZATION

Antisera for the passive immunization of tolerant fowls were prepared by injecting Brown Leghorn fowls two or three times with washed erythrocytes from 1 ml. turkey blood, or up to five times with 1 ml. whole White Leghorn blood. Antisera against White Leghorn blood were prepared and employed against the cells from an individual, but this restriction was not applied to antisera against turkeys. The antisera gave haemagglutination titres of 1 : 320–1 : 1280. For passive immunization, antiserum was injected intraperitoneally or subcutaneously in order to avoid shock, to which the fowls proved fairly susceptible. A dose of 1 ml. was chosen, as being large enough to eliminate tolerated erythrocytes rapidly, but small enough not to interfere with subsequent challenge. This dose, when given to fowls which had in their circulation tolerated erythroctyes labelled

with ^{51}Cr, caused the level of radioactivity in the blood to drop within a few minutes, and to fall below 5 per cent of the initial level within 2 to 6 hours. The lack of effect upon subsequent challenge was verified in an experiment in which normal fowls were injected with the standard dose of an anti-turkey antiserum at a titre of 1 : 640. Labelled turkey erythrocytes were then transfused at intervals. The initial lag-phase of elimination was missing to begin with, but by 5 days the pattern of elimination had returned to normal.
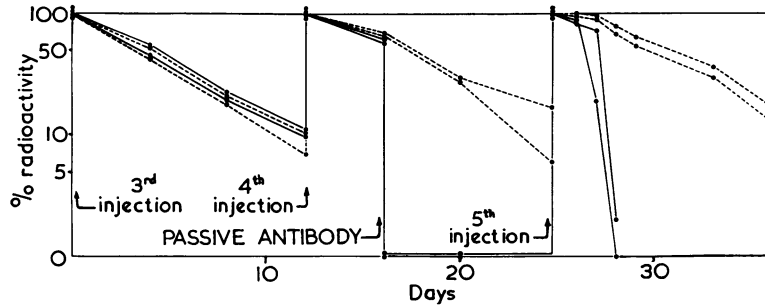

FIG. 3. Tolerance broken by passive immunization. Four chickens tolerant of their third transfusion of turkey erythrocytes; two (solid line) receive passive antibody, which eliminates the turkey cells received at the fourth injection, and hence breaks tolerance.

TABLE 4

THE EFFECT OF PASSIVE IMMUNIZATION ON TOLERANCE OF HOMOLOGOUS BLOOD

Days of age at last maintenance transfusion	Interval without ^{51}Cr in blood (days)	$T_{\frac{1}{2}}$ at penultimate maintenance transfusion — $T_{\frac{1}{2}}$ at challenge transfusion (days)
49	1–3 (control)	5.2–6.6; 4.8–4.5; 4.7–4.7; 4.5–2.8, 5.0; 4.3–5.1; 4.1–4.1
	11 (passive immunity)	6.0–5.3; 5.6–6.1; 5.2–4.5; 5.0–6.1; 4.9–6.0; 4.0–4.3; 3.7–5.7
83	0–2 (control)	6.1–10.1; 5.7–7.7; 5.1–6.1; 5.1–5.8; 4.5–8.2; 4.5–6.8; 4.3–4.9
	21 (passive immunity)	6.4–5.3; 6.0–4.6; 5.3–5.6; 3.0–5.0
125	11–16 (control)	8.2–5.0, 8.5; 6.1–4.8, 7.8; 5.6–5.5; 5.0–3.5, 7.7; 4.9–3.5; 4.5–3.2, 8.4
	38 (passive immunity)	10.1–1.5, 4.4; *7.7–1.5, 0.8*; *6.8–3.2, 1.5*; 5.8–2.3, 6.3; *5.3–1.5, 0.8*; *4.6—1.5, 1.5*
208	30 (passive immunity)	9.0–8.4; *8.5–ie3*; 7.8–6.7; 7.7–8.0; 6.3–5.5; 4.4–6.5

Fig. 3 illustrates the use of such an antiserum for the abolition of tolerance. Tolerance was first induced, as described before, in groups of fowls. Each group as it reached the time of test was split into two sub-groups, of which one was passively immunized, while the other was retained as a control (the control sub-group is missing from the oldest group shown in Table 4). Both sub-groups later received a challenge transfusion at the same time,

in order to test their state of tolerance. Passive immunization was carried out at a time when the foreign erythroctyes were known to be present in the circulation, as judged from the level of radioactivity; the procedure, as expected, always eliminated significant radioactivity within 24 hours. In consequence, the sub-group which received antiserum passed a longer period devoid of circulating antigen than the control, although both received an identical series of erythrocyte transfusions. The experiment serves to test the part played in maintaining tolerance by 'accessible antigen', i.e. antigen which can be reached or affected by circulating antibody.

TABLE 5

THE EFFECT OF PASSIVE IMMUNIZATION ON TOLERANCE OF TURKEY BLOOD

Days of age at last maintenance transfusion	*Interval without* ^{51}Cr *in blood (days)*	$T_{\frac{1}{2}}$ *at penultimate maintenance transfusion* — $T_{\frac{1}{2}}$ *at challenge transfusion (days)*
16	0 (control)	4.0–3.2; *3.8–ie4*; 3.2–3.5
	7 (passive immunity)	*4.0–ie3*
29	0 (control)	5.8–5.2; 3.4–4.4; 3.3–4.1; *3.3–2.5*; 3.2–4.3
	8 (passive immunity)	*4.3–ie3*; *4.3–ie3*; *4.0–0.8*; *3.8–ie3*; 3.5–ie3; 3.3–ie3
37	0 (control)	5.7–5.6; 5.6–5.3; 5.0–3.2; *4.9–2.2*; 4.7–4.8; 3.8–4.7; 3.6–4.9
	5 (passive immunity)	*4.6–1.1*; 4.1–4.1; *4.4–1.2*; *4.3–0.8*
44	0 (control)	4.0–3.5
	3 (passive immunity)	*4.9–0.8*

Tables 4 and 5 give the outcome of these tests. Only for tolerance of turkey erythrocytes could the intervals be arranged so that the control sub-groups retained the foreign cells in their circulation throughout the period of test. Tolerance of homologous erythrocytes needs a longer time for loss, and so the controls had to survive an interval without cells in order for the passively immunized bird to lose tolerance. In spite of this difficulty, it proved possible to arrange one unequivocal demonstration of loss, as shown by the 125-day-old group of Table 4, in which four out of six treated birds lost tolerance without any losses among the controls. Passive immunization has therefore been shown to cause a loss of tolerance, in the way to be expected if accessible antigen plays a part in maintaining tolerance. Indeed, the interval required for loss, as measured in this way, agrees well with the value obtained in the previous series of tests, in which erythrocytes were allowed to be removed from the circulation by the normal mechanism. From this agreement the conclusion may be drawn that erythrocyte antigens continue to contribute to the maintenance of tolerance for as long as they remain present in the circulation.

In this, as in the previous series of tests, reactivity developed sooner against turkey than homologous erythrocytes. Intervals after passive immunization of more than 5 days,

for instance, always abolished tolerance of turkey cells. Intervals of shorter duration than this could not be tested, because remains of the injected antiserum interfered with the outcome of the test. Nevertheless, the contrast with homologous erythrocytes is clear enough, for intervals of 11 days did not abolish homologous tolerance in birds of a comparable age-group. This relative instability no doubt helps to account for the difficulty that has been encountered in procuring heterologous tolerance, both in this and in previous work (Hašek, 1956). The observation appears to be of little theoretical importance, for all current theories of tolerance predict that tolerance should be lost soonest when the number of determinant groups is largest, provided that they are metabolized in the same way.

ADOPTIVE IMMUNITY

If tolerance of the present type represents a failure of immunologically competent cells to produce antibody, the condition should be abolishable by transplantation of competent cells from a normal animal. In an attempt to test this point, fresh heparinized blood was transfused from normal Brown Leghorns into recipients of the same strain which had been rendered tolerant of White Leghorn erythrocytes. The leucocytes of fresh heparinized blood are known to be immunologically competent, from their ability to give rise to a graft-versus-host reaction (Simonsen, 1957). In order to circumvent the homograft reaction against the transplanted leucocytes, an attempt was made at the same time to render mutually tolerant the donors and hosts.

Whole blood in quantities of 0.3–0.5 ml. was exchanged between pairs of Brown Leghorns, either 2–3 days before or within 12 hours after hatching. Skin-grafts were also exchanged within 24 hours of hatching, thus simultaneously enhancing antigenic stimulation and providing an indicator of the state of tolerance. To the exchanged blood was added an equal volume of irradiated blood of White Leghorn origin; each member of a Brown Leghorn pair received blood from a different, unrelated White Leghorn donor. Four further transfusions of irradiated White Leghorn blood were given after hatching, spaced according to the standard tolerance-inducing schedule. Immediately before the third transfusion, when the birds had attained an age of 4–5 weeks, 2.5 ml. of fresh heparinized blood was transfused between some of the Brown Leghorn pairs. Pairs were selected for this transfusion according to the following criteria: (1) the recipient exhibited tolerance of White Leghorn erythrocytes at the preceding transfusion, and (2) the host was tolerating the skin-graft from the donor, and vice versa. As a further check on the state of tolerance towards the transfused leucocytes, labelled erythrocytes from the donor of the fresh heparinized blood were transfused into the recipient after the last White Leghorn erythrocytes had been eliminated.

In the best of such experiments, only four pairs of birds met the requisite criteria of tolerance, and of these only three passed the further test of mutual tolerance towards one another's erythrocytes (even among these three, one leucocyte-donor reacted mildly against the erythrocytes). Each of these pairs is represented by one line in Table 6. A further twelve birds failed to meet the criteria of mutual tolerance between leucocyte-donor and host, but remained tolerant of White Leghorn erythrocytes throughout the experiment, and thus served as controls for the effect of adoptive immunization. The first two pairs shown in the table exhibited a reaction towards the White Leghorn erythrocytes, which could be interpreted as adoptive immunization. If this interpretation is accepted, the loss of tolerance appears to have taken place slowly. After the transfusion of leucocytes,

the first transfusion of White Leghorn erythrocytes was eliminated at the tolerant rate for 12 days, and was then eliminated faster. Not until the next transfusion was rapid elimination observed. These two instances provide the only evidence of adoptive immunity. As far as they go, they confirm the evidence of central failure of response and they make the loss of tolerance that has been brought about by absence of antigen appear to happen rapidly. They also show how difficult it is to investigate adoptive immunity in non-inbred animals.

TABLE 6

AN ATTEMPT AT ADOPTIVE IMMUNIZATION: THE EFFECT OF LEUCOCYTE-TRANSFER (BETWEEN MUTUALLY TOLERANT DONOR AND HOST) ON HOST'S TOLERANCE OF WHITE LEGHORN ERYTHROCYTES

Reactions of host:						Reactions of leucocyte-donor versus host's		
Versus White Leghorn Erythrocytes at			*Versus leucocyte-donor's*			*Erythrocytes*		*Skin*
			Erythrocytes		*Skin*			
$2\,(T_i)$	$3^*\,(T_i)$	$4\,(T_i)$	T	c		T	k	
4.4	3.6	1.5	24	2.6	+ +	12	4.3	+ +
3.3	2.9	1.3	22	5.7	+ +	24	2.3	+ +
3.2	4.7	4.6	24	2.3	+ +	22	5.7	+ +
3.8	5.6	4.8	5	—	+ +	4	—	+ +

* Leucocytes transferred immediately before this transfusion.

DISCUSSION

The experiments confirm the belief that an otherwise transient state of tolerance can be maintained by readministration of antigen. In young fowls, periodic readministration of antigen appears to be essential for the maintenance of tolerance towards erythrocyte antigens. From the evidence of passive immunization, the antigens of the erythrocytes appear to contribute towards the maintenance of tolerance for as long as they circulate in the blood. As the birds age, and at the same time receive a longer exposure to antigen, the durability of tolerance in the absence of circulating antigen increases. The increase takes place continuously; eventually, dependence on further antigen may be lost, but the question cannot be subjected to test in mortal animals. The interval devoid of circulating antigen which is needed for the loss of tolerance appears, according to the measurements made with homologous antigens, to be of the same order of size as the age of the bird. What has been measured in these experiments is a threshold of response and it is likely that other methods might give different values.

The progressive stabilization of tolerance can be regarded as an aspect of immunological maturation. From this point of view, the trend proceeds at a surprisingly slow pace. Chickens have developed the capacity to give an adult pattern of immune elimination, for example, before maturation in the present sense has been shown even to have begun. The overall growth of the chickens (shown in Fig. 2 of the preceding paper) provides a better guide to the durability of tolerance. The shortest time is needed for loss when the birds are growing most rapidly. This could be expected on theoretical grounds, if the rate of division of lymphoid cells also declines with age.

Some objections to these conclusions have already been discussed and answered (Mitchison, 1959). Erythrocytes from previous transfusions do not contribute enough

radioactivity to interfere with the interpretation of the challenge transfusion, nor is enough radioactivity absorbed to affect the immunological response. The inclusion of leucocytes among the transfused cells raises a more serious doubt, even though the blood was subjected to radiation. Two observations indicate that leucocytes made little contribution towards the maintenance of tolerance. First, viable cells — skin plus leucocytes — have already been shown to engender a long-lasting condition of tolerance, in contrast to tolerance of the present transient type. Second, passive immunization brings on the loss of tolerance in accordance with the time of elimination of erythrocytes from the circulation. The partial nature of the present type of tolerance raises another doubt; indeed, Billingham and Silvers (1960) suggest that antigen may be needed for the maintenance only of partial tolerance. However, the pre-existing level of tolerance appeared to influence the durability of tolerance in the present experiments only to a minor extent. As far as completeness is concerned, tolerance of the present type probably compares favourably with the tolerance of skin-grafts which was studied by Billingham and Silvers; the present evidence indicates that erythrocyte elimination offers the more sensitive method of detecting immunity.

A dependence of tolerance on the presence of antigen could be expected from previous work. Other erythrocyte antigens, for instance, are known to induce tolerance most effectively if given in repeated doses (Nossal, 1958; Hašek, 1960). The extra doses of antigen in some of these cases must have served to maintain, as well as to induce, tolerance. The most detailed studies have been made on soluble protein antigens, not always with concordant results. Both rabbits (Smith and Bridges, 1958) and mice (Dresser, 1961) require periodic readministration of foreign plasma proteins for the maintenance of tolerance, provided that these antigens are given in fairly small quantities. When antigens of the same type are given to rabbits in large quantities throughout the first months of life, tolerance of a much more long-lasting nature is engendered (Dixon and Maurer, 1955). It is in agreement with the present results that the older a rabbit is at the time when administration of antigen is discontinued, the longer does tolerance last.

Since the acquisition of tolerance can no longer be regarded as a once-and-for-all event, the theories which make this assumption are no longer required. The reactive cells of the embryo, for instance, need no longer be assumed to undergo a genetic change under the influence of antigen (Brent, 1958). Indeed, the principal argument for a genetical theory of tolerance has now been lost. An attractive way of accounting for the need for further antigen is to assume, as was first suggested by Lederberg (1958), that the presence of antigen serves to block the maturation of stem-cells into mature cells. Lederberg assumed that the block consisted of a selective destruction of cells endowed with the capacity for making a specific antibody; the precise nature of the block is, of course, unknown and other mechanisms can be imagined (Medawar, 1960). According to the stem-cell theory, then, cellular maturation occupies the interval between the loss of antigen and the gain of reactivity. This theory is in accordance with the present data, except at one point: brief exposure of mature animals to antigen, even at high concentrations, would not be expected to paralyse the immune response. Yet the experiments on eight-day-old chickens, described in the preceding paper, appear to show that this can be done. The stem-cell theory can be retained only by making one of the following assumptions: (1) mature cells can be inhibited, but at a higher threshold of antigen concentration than stem cells, or (2) a second and distinct mechanism operates in paralysis by overdose of antigen. On the other hand the loss of tolerance can equally well be accounted for without

recourse to stem-cells, by the loss of antigen from some controlling site within the mature reactive cell.

Whichever of these theories is adopted, the presence of antigen in some controlling position — the intracellular 'tolerance-site' — may be required for inhibition. This has been a popular assumption (Terres and Hughes, 1959), yet certain difficulties have to be faced. In the first place, the present data offer no support for the hypothesis that the tolerance-site is identical with the immunity-site, with inhibition resulting simply from an excess of antigen. For the loss of tolerance appeared to leave birds in a reactive condition, but not in the state of active immunity which would be predicted by this hypothesis. In the second place, a wide variation has been observed in the time required for gain of reactivity. The difficulty is most acute in the case of old birds which need months or years without circulating antigen, before tolerance is lost. When it is recalled that their previous treatment probably provided each lymphoid cell with only a few molecules of antigen, it becomes hard to believe that so long an interval is needed just for the degradation of antigen.

ACKNOWLEDGMENTS

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