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Survival of H-2 Incompatible Mouse Erythrocytes in Untreated and Isoimmune Recipients

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Summary. ^{51}Cr labelled strain A erythrocytes transfused into various H-2 incompatible mouse strains (A.CA, A.BY, A.SW, C3H and DBA/2) were not eliminated more rapidly than after transfusion into compatible strain A recipients. In contrast, C57BL and, in particular, C57L mice often eliminated strain A red cells at an accelerated rate. Attempts were made to analyse the mechanism responsible for red cell survival in strains other than C57L and C57BL. Red cells surviving normally in H-2 incompatible recipients nevertheless induced the formation of humoral antibodies directed against themselves. Transfusion of erythrocytes into actively immunized recipients resulted in the rapid elimination of one fraction of the population, whereas the surviving fraction was eliminated at the same rate as in isologous hosts. Analogous results were obtained after transfusion into H-2 incompatible recipients passively immunized with humoral anti-H-2 antibodies. Large doses of antibodies or antibodies plus passively transferred guinea-pig serum (0·5 ml. per recipient) as a source of complement did not increase the proportion of eliminated red cells. Passively transferred humoral antibodies were capable of eliminating a fraction of the red cells only if given before or during the first 4 days after transfusion; they had no such effect if given later. The failure of antibodies to eliminate all transfused cells was not caused by the existence of a fraction of antibody-resistant cells, since the antibodies caused complete lysis of the red cells *in vitro* in the presence of complement. It is suggested that a combination of antibodies and some host factors induce a change in the red cells that makes them resistant against isoimmune destruction.

INTRODUCTION

The isoimmune reactions developing as a consequence of transplantation of cells and tissues into genetically different recipients efficiently prevent the survival of most grafts. In the mouse the H-2 isoantigen system is responsible for the most efficient barrier against the prolonged survival of allogeneic transplants. H-2 isoantigens are widely distributed in the tissues of the mouse and are also present on erythrocytes. Certain tissues are exceptional in their transplantation behaviour, however, in being able to survive in H-2 incompatible recipients. Cartilage is one example: it immunizes allogeneic hosts but nevertheless survives for prolonged periods of time. The cells seem to be protected from the homograft reaction by the mucopolysaccharide matrix (Stjernswärd, 1964). Neoplastic cells of certain types belong to this category as well. Such so-called non-specific

tumours can grow in histoincompatible recipients, although they induce an intense immune response. Their cells are characterized by a low concentration of surface isoantigens which makes them apparently comparatively refractory to the homograft reaction (E. Möller, 1964).

Recent studies have indicated that erythrocytes belong to the category of cells capable of unhampered survival in H-2 incompatible recipients (Goodman and Smith, 1961; Möller, 1963b; Erickson, Herzenberg and Goor, 1964). The responsible mechanism is not known. Certain properties seem to differentiate mouse red cells from other cell types, however. Thus, Medawar (1959) found that red cells were incompetent to immunize H-2 incompatible recipients against subsequent skin grafts. It seems nevertheless established that red cells contain H-2 antigens and react with humoral agglutinating and haemolytic isoantibodies *in vitro*.

The present study is concerned with the behaviour of erythrocytes after transfusion into H-2 incompatible recipients in an attempt to elucidate some of the factors responsible for their survival. Particular attention has been given to the effect of active and passive immunization on the elimination of incompatible erythrocytes.

MATERIAL AND METHODS

Mice of the following inbred strains were used: A/SnKl (*H*-2^a), A.CA/Kl (*H*-2^f), A.BY/Kl (*H*-2^b), A.SW/Kl (*H*-2^s), C3H/Kl (*H*-2^k), DBA/2/Kl (*H*-2^d), C57BL/Kl (*H*-2^b) and C57L/Kl (*H*-2^b). In some cases F_1 hybrids between two of these strains were used.

Isotope Labelling of Erythrocytes

Red cells were taken from the retro-orbital sinus, suspended in citrate (0·1 M) solution and washed twice in B(alanced) S(alt) S(olution). The cells were resuspended in an equal volume of BSS and ^{51}Cr was added in the form of Na_2CrO_4. The mixture was incubated at 37° for 30 minutes and thereafter washed three times with 10 ml. BSS each time. A 10 per cent v./v. suspension was made up in BSS and 0·2 ml. of this suspension was injected intravenously within 2 hours after labelling. The amount of chromium added to the cells never exceeded 10 μg./ml. red cells. In most cases each recipient received between 5 and 15 μc. ^{51}Cr, but in cases where strong radioactivity was needed the dose was increased up to 50 μc. per recipient.

Red cell survival was followed by bleeding the mice from the retro-orbital sinus at 3–6 day intervals; 0·05 ml. blood was withdrawn with a micropipette and added to 2 ml. of water.

Radioactivity measurements were performed in a well-type scintillation detector (Tracerlab); they were continued for 10 minutes or until 100,000 counts had been recorded. All counts given in the text have been corrected for radioactive decay and background.

Serological Procedures

Haemagglutination was performed according to the technique of Gorer and Mikulska (1954) as modified by Stimpfling (1961) and read as described previously (Möller, 1961). Haemolytic tests were performed according to the method of Hildemann (1957) with slight modifications: 0·2 ml. antiserum was serially diluted in BSS containing 1 per cent gelatin and 0·1 ml. of a 2 per cent red cell suspension and 0·1 ml. guinea-pig serum diluted 1/2 was added. The same lot of lyophilized guinea-pig serum (Sclavo, Italy) was used

throughout the investigation. After incubation for 1 hour at 37° the tubes were centrifuged at 3000 *g* for 2 minutes and 0·2 ml. of the supernatant was withdrawn for radioactive determinations. In all experiments three controls were included: one in which antiserum was omitted and replaced with BSS, one in which complement was omitted, and a third in which the red cells were suspended in water. For calculations of the degree of haemolysis the last control was taken as the value for 100 per cent lysis and the control with complement in the absence of immune serum as the value for 0 per cent.

RESULTS

SURVIVAL OF STRAIN A ERYTHROCYTES IN VARIOUS MOUSE STRAINS

Strain A erythrocytes labelled with ^{51}Cr were transfused by the intravenous route into untreated adult isologous recipients (strain A or F_1 hybrids with A as one parent) and into various incompatible strains (A.CA, A.BY, A.SW, C3H, DBA/2, C57L and C57BL). The survival of red cells was measured in 0·05 ml. whole blood samples by repeated bleedings at 3–6 day intervals. Activity on the first bleeding (day 0 or 1) was taken as the value for 100 per cent survival. Each experiment was performed with groups of three to four mice of each genotype and altogether 100 mice were used. The elimination curves of the transfused A red cells from the compatible recipients were linear on a semilog scale. Ninety-five per cent of the cells were eliminated after 29 days (Table 1). The survival times were

TABLE 1
SURVIVAL TIMES* OF STRAIN A ERYTHROCYTES LABELLED WITH ^{51}Cr IN VARIOUS H-2 INCOMPATIBLE RECIPIENTS

Recipients	*No. of mice*	50 *per cent survival times of transfused cells (days)*	5 *per cent survival times of transfused cells (days)*	*Type of elimination*
A or A hybrids	30	5, 5, 6, 6, 6, 7, 7, 7, 7, 7, 8, 9, 9, 9, 9, 9, 9, 10, 10, 10, 10, 10, 10, 10, 11, 11, 11, 11 Mean = 8·6	20, 21, 25, 25, 26, 27, 27, 28, 28, 29, 29, 29, 29, 30, 30, 30, 31, 31, 31, 32, 32, 33, 33, 33, 33, 33, 33, 34, 34, 34 Mean = 29·4	Normal 30/30
A.CA	18	5, 6, 6, 6, 6, 7, 7, 7, 7, 7, 7, 7, 7, 8, 9, 9, 9, 10 Mean = 7·2	20, 22, 22, 23, 24, 25, 26, 27, 29, 29, 30, 30, 30, 30, 31, 31, 31, 31 Mean = 27·3†	Normal 18/18
A.BY	9	5, 6, 7, 7, 8, 8, 8, 8, 9 Mean = 7·3	21, 23, 25, 27, 28, 28, 30, 30, 31 Mean = 27·0†	Normal 9/9
A.SW	7	7, 7, 7, 7, 7, 8, 8 Mean = 7·3	25, 26, 26, 27, 29, 30, 31 Mean = 27·7†	Normal 7/7
C3H	10	7, 8, 8, 8, 8, 8, 8, 9, 9, 10 Mean = 8·3	29, 29, 30, 30, 30, 32, 32, 33, 33, 34 Mean = 31·2†	Normal 10/10
DBA/2	6	8, 9, 9, 10, 10, 11 Mean = 9·5	30, 31, 32, 34, 35, 35 Mean = 32·8†	Normal 6/6
C57BL	9	5, 5, 6, 6, 6, 6, 6, 6, 8 Mean = 6·1	21, 23, 26, 27, 29, 30, 31, 32, 32 Mean = 27·9†	Accelerated 3/9
C57L	11	4, 4, 5, 5, 5, 6, 7, 7, 7, 8, 10 Mean = 6·2	10, 10, 11, 11, 14, 16, 19, 19, 20, 23 30 Mean = 16·6‡	Accelerated 10/11

* The activity present in the first bleeding after transfusing (day 0 or 1) was taken as 100 per cent survival.
† There is no statistically significant difference ($P > 2$) in the mean survival time as compared to isologous recipients.
‡ There is a highly significant difference ($P < 0·001$; $t = 22·9$; d.f. = 39) in the mean survival time as compared to isologous recipients.

shorter than reported previously by Goodman and Smith (1961) presumably because each recipient was bled repeatedly; each bleeding removes about 5–10 per cent of the transfused cells. This is of minor importance in the present context since the intention was to compare the cell survival in compatible and incompatible recipients.

The elimination pattern of transfused strain A red cells in H-2 incompatible recipients varied with the recipient strain. In strains A.CA, A.BY, A.SW, C3H and DBA/2 the cells were eliminated in exactly the same way as in isologous hosts and there were no signs of immune destruction. In strains C57L and C57BL the cells disappeared faster as a rule. This was most clearly seen in C57L, where an increased rate of elimination was observed after 5–10 days in nine out of eleven recipients. In C57BL there was more individual variation: in three out of nine cases the rate was slightly faster than in the compatible A strain but in six animals there was no increase. The results are summarized in Table 1 and some typical elimination curves are illustrated in Fig. 1.

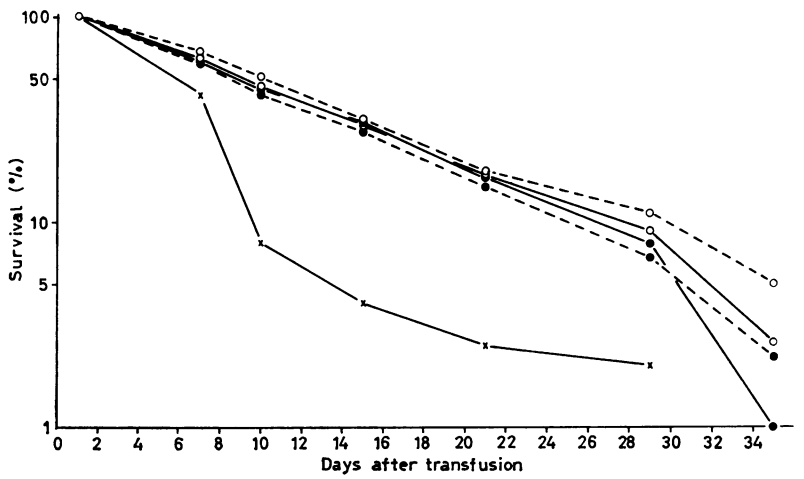

FIG. 1. Elimination of intravenously injected ^{51}Cr labelled strain A erythrocytes in A (○——○), A.CA (●——●), DBA/2 (○ - - - ○), A.BY (● - - - ●) and C57L (×——×) recipients. Each point represents the mean value of four mice.

Thus, with the exception of C57L and C57BL, strain A red cells survived in all H-2 incompatible strains in exactly the same way as in the strain of origin. In the two exceptional strains the elimination process was more rapid, but could not be regarded as typical of immune elimination; the disappearance was usually slow and incomplete compared to the results obtained in other species (Fig. 1). The increased elimination in the C57BL and C57L genotypes could not be ascribed to a particularly strong antigenic discrepancy between the donor and the recipients ($H\text{-}2^a$ and $H\text{-}2^b$, respectively), since no increased elimination was found in A.BY which is also $H\text{-}2^b$.

It has been claimed that transfusion of incompatible cells by the intravenous route may induce exceptional immunological reactions leading to prolonged survival of subsequent grafts (Billingham and Sparrow, 1955). Experiments were performed, therefore, to investigate whether the survival of strain A red cells in incompatible recipients was dependent on the route of transfer or could also be demonstrated upon intraperitoneal injection. ^{51}Cr labelled strain A red cells were injected intraperitoneally into groups of A.CA recipients and into compatible (A × A.CA)F_1 hybrids and their elimination was followed by

repeated bleedings. It was found that the injected cells entered into the general circulation and could already be detected after 1 hour, with maximum activity after 24 hours. Subsequently the cells were eliminated at a constant rate, which was the same in compatible and incompatible hosts (Fig. 2).

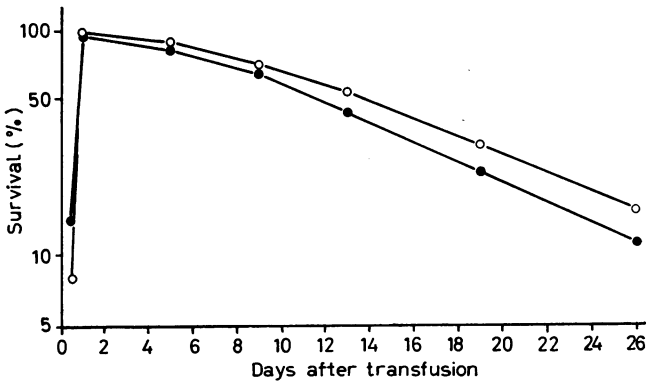

FIG. 2. Elimination from the blood of intraperitoneally injected ^{51}Cr labelled strain A erythrocytes in (A × A.CA)F_1 (○) and A.CA (●) recipients. Each point represents the mean value of three recipients.

The failure of most H-2 incompatible recipient strains to show immune elimination of strain A erythrocytes is in marked contrast to their efficient rejection of grafted strain A skin, lymphoid cells and tumour cells of various histological types. The survival of the red cells cannot be ascribed to any absence of H-2 antigens, because the injected cells could be easily agglutinated and haemolysed *in vitro* by anti-A sera produced in any of the recipient strains prior to transfusion.

It seemed possible that the failure of the hosts to eliminate the incompatible red cells was due to an insufficient immunogenicity of the erythrocytes, in accordance with the findings of Medawar (1959), who showed that red cells purified from contaminating white cells could not detectably sensitize incompatible hosts against subsequent skin grafts. Although this possibility seems less likely in the present experiments, since no attempts were made to eliminate white cells, it was investigated whether the incompatible recipients became sensitized to the transfused strain A cells by studying the development of agglutinating antibodies at various times after injection. It was regularly found that A.CA recipients transfused with strain A red cells synthesized humoral isoantibodies capable of agglutinating A red cells *in vitro*. The titres were usually low compared to those obtained after inoculation of 10–20 × 10^6 strain A lymphoid cells (Möller, 1963a) and varied between 1/2 and 1/256; they were detectable after 5 days and persisted during the 26-day period tested (Fig. 3). The results demonstrate that the recipients do react against the transfused incompatible red cells by the production of humoral isoantibodies; the survival of the cells cannot be explained, therefore, by a central failure of the immune response, such as would be expected if tolerance-like phenomena were responsible.

The paradoxical situation thus exists that incompatible erythrocytes and isoantibodies directed against them are circulating in the host without deleterious effects to the cells.

TRANSFUSION OF STRAIN A ERYTHROCYTES INTO ACTIVELY AND PASSIVELY IMMUNIZED H-2 INCOMPATIBLE RECIPIENTS

It was studied whether a pre-existing immunity would affect the survival of transfused

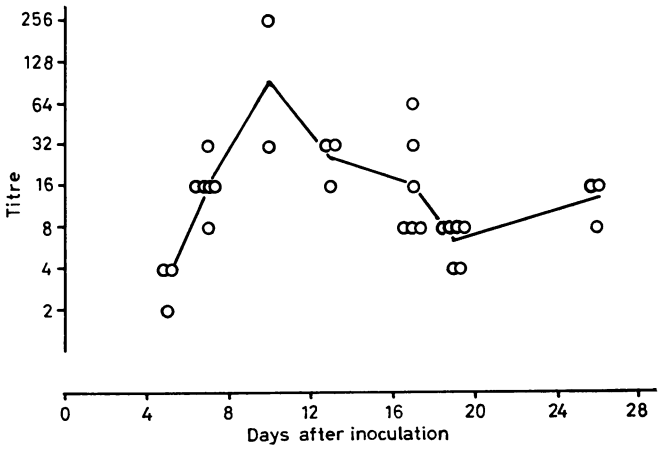

FIG. 3. Production of haemagglutinating antibodies against strain A red cells by A.CA recipients subsequent to transfusion with A erythrocytes.

incompatible red cells. Labelled strain A red cells were transfused intravenously into groups of A.CA recipients (altogether twenty-six), that had been pre-immunized against A lymphoid cells by intraperitoneal inoculations of living cells. The number of inoculations varied in different experiments between one and ten, given at weekly intervals, the last 6 days prior to the experiment. At the time of transfusion all animals had agglutinating and haemolysing antibodies directed against A cells.

All experiments except one gave similar results: a proportion of the strain A red cells was eliminated from the circulation within 1 hour (Table 2) whereas the surviving frac-

TABLE 2

ELIMINATION OF ^{51}Cr LABELLED STRAIN A ERYTHROCYTES IN A.CA RECIPIENTS ACTIVELY IMMUNIZED AGAINST A LYMPHOID CELLS

Recipients	*No. of injections with A lymphoid cells**	*Elimination of transfused erythrocytes (% of the injected dose)†*	*Survival of non-eliminated erythrocytes*
A.CA	One i.p.	32·0, 36·9, 40·5	Normal
A.CA	One s.c.	33·8, 38·9, 65·6	Normal
A.CA	One s.c.	38·2, 37·3, 43·9	Normal
A.CA	Five i.p.	55·1, 57·5, 62·2	Normal
A.CA	Ten i.p.	49·4, 70·3, 79·3	Normal
A.CA	Six i.p.	77·0, 78·4, 86·7, 88·4	Normal
A.CA	Six i.p.	37·3, 56·9, 70·2, 88·2	Normal
A.CA	Six i.p.	92·6, 93·7, 94·1	Complete destruction of the cells within 10 days

* Between 30 and 100×10^6 spleen and lymph node cells were injected, the last 6 days prior to the experiment.

† The degree of elimination was calculated from the first time of bleeding after transfusion (after 1 hour or 24 hours). Non-immunized A.CA recipients served as controls.

tion was completely unaffected and became eliminated in parallel with the cells transfused into untreated A.CA hosts or into compatible (A × A.CA)F_1 hybrids (Fig. 4).

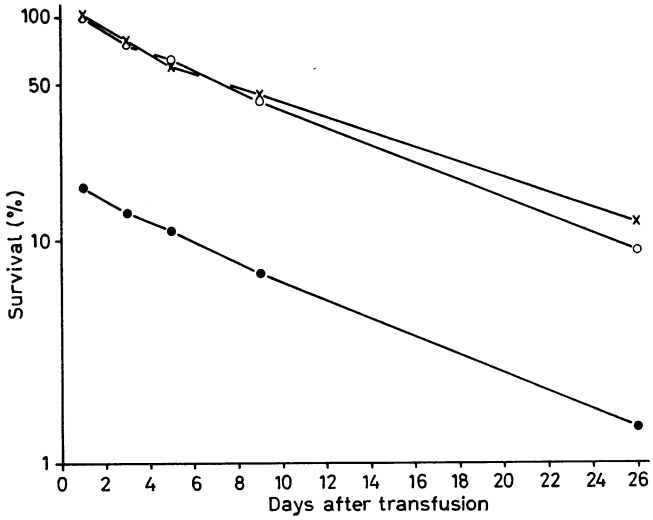

FIG. 4. Survival of ^{51}Cr labelled strain A red cells in (A × A.CA)F_1 (×), in untreated A.CA (O) and in A.CA recipients which had been given six intraperitoneal injections with strain A lymphoid cells, the last given 6 days prior to the experiment (●). Each point represents the mean value of four mice.

The initial elimination varied between 32 per cent and 88 per cent of the injected cells in different experiments, the mean value being 61.5 per cent (Table 2). The variation was caused more by differences between various experiments than by variation between different individuals within the same experiment. The number of immunizations and the route of immunization did not influence the extent of the initial elimination to any detectable degree.

In the exceptional experiment mentioned above strain A red cells were transfused into three A.CA recipients which had been given three weekly injections of A lymphoid cells. This resulted in a rapid elimination of the red cells and only about 5 per cent was left after 1 day; the rest disappeared within the next 10 days. This finding could not be reproduced in subsequent tests.

Red cells of strain A origin were sensitive *in vitro* to the haemolytic effect of A.CA anti-A serum in the presence of guinea-pig complement. The degree of haemolysis varied with different lots of antisera; usually 100 per cent of the cells were lysed, but with some sera the degree of lysis was less (80–90 per cent). Since the A.CA recipients contained high titres of haemolytically efficient antibodies in the above described experiments, but were nevertheless incapable of eliminating the foreign cells *in vivo*, a study was made on the effect of passively transferred isoantibodies on the elimination of transfused incompatible A red cells.

Undiluted antiserum (0·1 ml.) was given intraperitoneally about 30 minutes prior to the intravenous transfusion of the ^{51}Cr labelled strain A red cells, or, alternatively the antiserum was mixed *in vitro* with the red cells and the mixture was injected intravenously. The results were similar with both procedures and they will be reported together.

It was found that passively transferred humoral isoantibodies directed against A cells and produced in strains A.CA, A.BY, C3H, DBA/2, C57L and C57BL caused a rapid initial elimination of a fraction of the red cells. The proportion of eliminated erythrocytes varied in different experiments between 5 and 97 per cent (mean elimination in 132

TABLE 3

EFFECT OF PASSIVELY TRANSFERRED ISOANTISERUM ON THE *in vivo* ELIMINATION OF TRANSFUSED STRAIN A ERYTHROCYTES

Recipients	*No. of mice*	*Pretreatment of recipients with*	*Mean destruction of transfused red cells (% of injected dose)*	*Removal of transfused erythrocytes (% of injected dose)*									
				Distribution of mice									
				0–9·9	10–19·9	20–29·9	30–39·9	40–49·9	50–59·9	60–69·9	70–79·9	80–89·9	90–99·9
A.CA	100	A.CA anti-A serum*	51·4		4	6	17	18	23	22	7	2	1
A.BY	9	A.BY anti-A serum*	35·9	1		3	2		1	2			
C3H	6	C3H anti-A serum*	36·5	1		1	1	1	2				
DBA/2	3	DBA/2 anti-A serum*	16·2	1	1		1						
C57L	9	C57L anti-A serum*	44·4			1	1	3	4				
C57BL	5	C57BL anti-A serum*	53·8				1	1	1	2			

* The antiserum was injected intraperitoneally into the recipients 30 minutes prior to transfusion of the red cells or was mixed with the red cells *in vitro*. Usually 0·1 ml. undiluted serum was used, but the table also includes experiments where 0·3, 0·7 and 0·8 ml. serum was given.

mice = 48·5 per cent). There was no major difference between the effect of antisera produced in different strains and the titres of the sera did not detectably influence the proportion of eliminated cells. The surviving cells were completely unaffected and the slope of the elimination curves paralleled those found in untreated recipients. The results were thus analogous to those obtained in actively immunized hosts, illustrated in Fig. 4. The experiments are summarized in Table 3.

It could be excluded that the red cells survived because of insufficient amounts of passively transfused humoral isoantibodies. Several experiments were performed with different antibody doses and it was demonstrated that increased amounts of antibodies (up to 0·8 ml. per recipient) did not eliminate a larger fraction than 0·1 ml. antiserum. When less than 0·1 ml. undiluted antiserum was employed, however, the degree of destruction varied with the antibody dose. As illustrated in Table 4, 0·1 ml. of an A.CA anti-A

TABLE 4

EFFECT OF VARIOUS ANTISERUM DOSES ON THE ELIMINATION OF STRAIN A ERYTHROCYTES

Exp. no.	*Recipients*	*Antiserum*		*Elimination of transfused erythrocytes (% of dose injected)*
		Specificity	*Dose*	
1	A.CA	A.CA anti-A	0·1 ml. diluted 1/160	5·1, 10·9, 32·1 Mean = 16·0
			0·1 ml. diluted 1/20	34·7, 35·1, 45·1 Mean = 38·3
			0·1 ml. diluted 1/1	36·3, 37·1, 40·8 Mean = 38·1
			0·4 ml. diluted 1/1	30·0, 37·2, 42·7 Mean = 36·6
2	A.BY	A.BY anti-A	0·1 ml. diluted 1/50	0·5, <0, <0 Mean = <0
			0·1 ml. diluted 1/10	27·8, 47·7, 49·0 Mean = 41·5
			0·1 ml. diluted 1/1	57·2, 62·1, 62·5 Mean = 60·6

serum diluted 1/160 had a small but detectable effect on the removal of A cells in otherwise untreated A.CA recipients. This effect increased to an apparently maximal value when the antiserum dilution was decreased to 1/20. Larger antiserum doses did not increase the degree of elimination further.

The possibility that the persistence of the radioactive label in the circulation of the recipients was due to transfer of ^{51}Cr to host cells could be excluded by the demonstration that all radioactivity was bound to the originally injected cells. Samples of blood from A.CA recipients transfused with ^{51}Cr labelled strain A cells were removed at various intervals during the first 4 days after transfusion and treated *in vitro* with A.CA anti-A sera and guinea-pig complement. All radioactivity in the sample was released into the supernatant demonstrating that the A cells contained all the radioactive label. Furthermore, injection of lysates obtained by adding distilled water to ^{51}Cr labelled cells did not result in labelling of the host cells, and all radioactivity disappeared from the circulation within 1 day.

It seemed possible that insufficient complement levels *in vivo* might be responsible for the low efficiency of humoral antibodies in eliminating the incompatible cells. This could be excluded by the repeated finding that injection of isoantibody and 0·5 ml. undiluted guinea-pig serum, fully active against isoantibody-sensitized A erythrocytes *in vitro* failed to increase the proportion of eliminated cells in comparison to that obtained with antibody alone (Table 5).

The possibility existed that some other host functions required for red cell destruction were limiting, with partial removal of the antibody-treated erythrocytes as a result. If

TABLE 5

EFFECT OF COMPLEMENT ON THE ANTIBODY-INDUCED ELIMINATION OF STRAIN A ERYTHROCYTES

Exp. no.	*Recipients*	*Pretreatment of recipients*	*Elimination of transfused erythrocytes (% of injected dose)*
1	A.BY	A.BY anti-A serum*	20·6, 26·1, 31·1 Mean = 25·9
		A.BY anti-A serum and complement†	10·0, 23·9, 34·5 Mean = 22·8
2	A.CA	A.CA anti-A serum	24·8, 35·9, 46·2 Mean = 35·6
		A.CA anti-A serum and complement	30·3, 33·3, 34·4 Mean = 32·7
3	A.CA	Complement	2·7, 9·7, 9·9 Mean = 7·4
		A.CA anti-A	37·0, 39·2, 50·9 Mean = 42·4
		A.CA anti-A serum and complement	23·5, 32·7, 39·9 Mean = 32·0

* 0·1 ml. undiluted antiserum was injected intraperitoneally 30 minutes prior to transfusion.

† 0·5 ml. undiluted guinea-pig serum was injected intraperitoneally 5 minutes after the antiserum.

this were the case, it would be expected that a second injection of antibody-sensitized red cells shortly after a first one would not be followed by the elimination of the usual fraction of the cells. This possibility could not be verified experimentally, however. Unlabelled strain A red cells were transfused into antibody-treated (A.CA anti-A serum) A.CA recipients and 1 hour later ^{51}Cr labelled A red cells were injected into the same hosts. It was found that a proportion (71·6 per cent) of the latter cells were eliminated after 1 hour; an equally large fraction (72·8 per cent) of ^{51}Cr labelled cells were eliminated in antibody-treated but otherwise untreated A.CA hosts. Thus, recipients that had previously eliminated a fraction of transfused isoantibody-sensitized cells were fully competent to eliminate a similarly large proportion of subsequently transfused cells.

EFFECT OF PASSIVELY TRANSFERRED ISOANTIBODIES ON THE ELIMINATION OF INCOMPATIBLE RED CELLS AT VARIOUS TIMES AFTER TRANSFUSION

The absence of immune elimination of H-2 incompatible red cells in most recipients appeared to be due to some properties of the red cells rather than to the failure of the hosts to develop immunological reactions, as evidenced by the production of humoral antibodies subsequent to the transfusion of incompatible red cells. Simple explanations, such as the absence of the relevant isoantigenic receptors on the red cells, could be excluded by the finding that complete lysis occurred *in vitro* after treatment of the red cells with isoantisera and complement. Since all cells appeared to be equally susceptible to haemolytic isoantibodies *in vitro* it is unlikely that a fraction was resistant. The survival of a substantial proportion of the red cells after antibody-treatment *in vivo* cannot be explained, therefore, by the existence of preformed resistant variants. The results are compatible with the interpretation that antibodies induce a change making red cells refractory to immune destruction. It appears possible that the absence of immune elimination in untreated incompatible recipients is due to the induction of a similar change by the action of actively produced antibodies. If this is so, it would be expected that passively transferred antibodies would not eliminate the usual proportion of transfused incompatible red cells, provided that

these had been present in the untreated foreign hosts for such a long period that active antibody production had been initiated. Prior to the development of antibody production the transfused cells should, however, be still sensitive to the effect of passively transferred antibodies. Experiments were performed to test this possibility.

Strain A red cells were transfused into untreated A.CA recipients; at various intervals groups of three mice were injected with 0·1 ml. undiluted A.CA anti-A serum. All mice were bled immediately prior to the antibody injection and also 1 hour later. It was found that passively transferred antibodies were capable of eliminating a proportion (47–66 per cent) of the red cells during the first 3 days after transfusion; the same proportion was removed when antibodies were given before transfusion. During the following days the effect of the passively transferred antibodies became gradually less pronounced and after 7–9 days only 10 per cent and 7 per cent respectively were eliminated (Fig. 5). Thus,

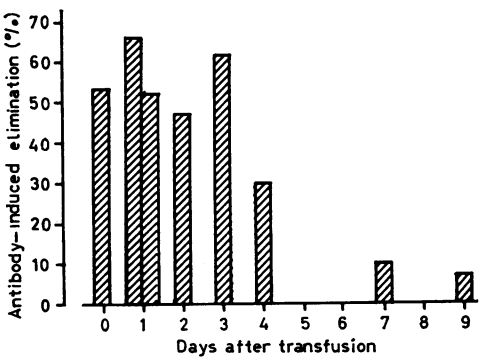

FIG. 5. Effect of A.CA anti-A serum on the elimination of ^{51}Cr labelled strain A erythrocytes at various times after transfusion into A.CA recipients. The mice were bled immediately before antiserum injection and the degree of elimination was determined 1 hour later. Each bar represents the mean value of three mice.

passively transferred antibodies affected transfused red cells considerably less when they had been circulating in the foreign hosts for more than 4 days; this change coincides with the appearance of actively produced humoral antibodies directed against the transfused cells.

TISSUE DISTRIBUTION OF ANTIBODY-ELIMINATED H-2 INCOMPATIBLE RED CELLS

Strain A red cells were completely lysed *in vitro* by an A.CA anti-A serum in the presence of guinea-pig complement, but were nevertheless only partially eliminated *in vivo* by the same antiserum. It seemed possible that different mechanisms of red cell destruction were operating *in vivo* and *in vitro*. It was investigated, therefore, whether antibodies eliminated foreign red cells by intravascular haemolysis or by other mechanisms. It would be expected that intravascular haemolysis should lead to release of the radioactive label into the serum. ^{51}Cr labelled strain A red cells were transfused into A.CA recipients, followed by the intraperitoneal injection of an A.CA anti-serum 15 minutes later, or, alternatively, the antibodies and the red cells were mixed *in vitro* prior to injection. Control groups did not receive the antibody injection. Two mice of each group were killed at various intervals during the subsequent hours and 0·05 ml. of blood was taken from each mouse and suspended in citrate solution. The red cells were spun down and the

supernatant was withdrawn for radioactivity measurements. Prior to the measurement of the activity in the red cells they were washed three times. In addition, the liver and spleen and, in some experiments, the kidney, was taken from each mouse and used directly for radioactivity determinations.

It was regularly found that injection of antibodies caused a rapid increase of the radioactivity of the liver and spleen, whereas no increase was observed in plasma (Fig. 6). The

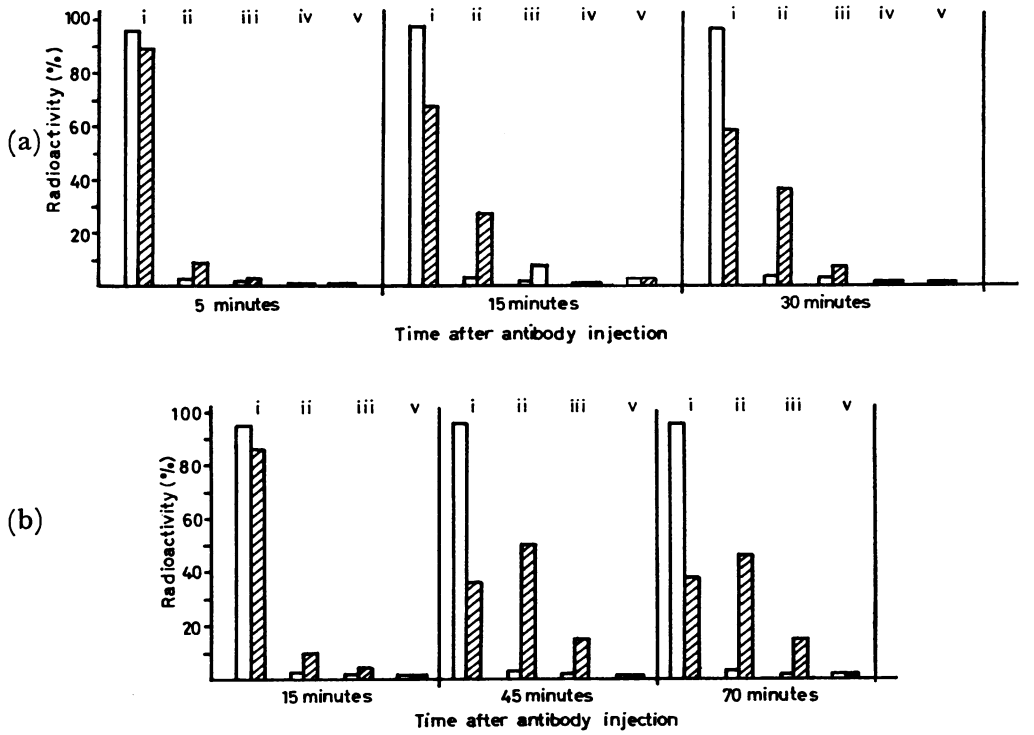

FIG. 6. Tissue distribution of ^{51}Cr labelled strain A red cells in untreated A.CA recipients (open columns) and in antiserum-treated (A.CA anti-A serum) A.CA recipients (shaded columns) at various intervals after the serum injection. In experiment (a) the antiserum and the red cells were mixed *in vitro* prior to transfusion and in experiment (b) the antibodies were injected intraperitoneally 1 hour after red cell transfusion. Each bar represents the mean value of two mice. (i) Erythrocytes; (ii) liver; (iii) spleen; (iv) kidney; (v) plasma.

effect was rapid and could be detected already after 5 minutes. Two typical experiments are illustrated in Fig. 6. It appears that antibodies do not eliminate the target red cells by lysing them *in vivo*, but rather by acting as opsonins for subsequent phagocytosis in the liver and the spleen.

DISCUSSION

Immune elimination of blood group incompatible erythrocytes after transfusion into recipients lacking preformed antibodies occurs in many species, such as man (Mollison, 1961), dog (Swisher and Young, 1954; Stohlman and Schneiderman, 1956), sheep (Drury and Tucker, 1958) and fowls (Mitchison, 1962). Elimination usually precedes the appearance of detectable serum antibodies and, therefore, destruction of incompatible

cells *in vivo* is regarded as an unusually sensitive test for the detection of immunological reactions mediated by humoral isoantibodies (Chaplin, 1959; Mitchison, 1962). However, the elimination of incompatible erythrocytes does not occur in all species or in all antigenic systems. Absence of immune destruction after transfusion of red cells into recipients not containing preformed antibody is often found with human antigenic systems other than Rh (Mollison, 1961; van Loghem, van der Hart, Engelfriet and van de Wiel, 1962), in rats (Smith, Odell and Caldwell, 1959) and in other species.

Transfusion of incompatible erythrocytes into recipients containing specific isoantibodies, occurring naturally or as a result of active or passive immunization, leads to destruction of the injected cells in all species investigated as a rule. The rate of elimination might be influenced by the type of the antigenic difference between donor and host, by the amount of antibodies present, and by the type of the antibodies (for discussion see Mollison, 1961). Survival of cells in the presence of antibodies has been observed in rare cases. Thus, Mollison (1961) observed only partial elimination of transfused human cells. Greendyke and Chorpenning (1959) reported a case of normal red cell survival in the presence of isoantibodies (anti-Lu^a) and Swisher and Young (1954) encountered a similar phenomenon in dogs. The results were ascribed to heterogeneity of the antigen distribution within the red cell population and was mostly encountered with 'weak' antigens. It was suggested that a certain minimal amount of antibody must be bound by the red cells to permit their accelerated destruction and that this requires the saturation of a certain minimal number of antigenic sites (Mollison, 1961). Drury and Tucker (1958) observed survival of sheep red cells in the presence of antibodies in a few cases. However, their experiments concerned the R antigen, which is soluble and becomes attached to the red cells. They discussed the possibility that the red cells lost their antigen *in vivo* and became compatible as a result.

A phenomenon resembling the one studied in the present paper has been observed after transfusion of autologous rabbit erythrocytes coated with bacterial polysaccharides. Such cells are rapidly lysed intravascularly if transfused into recipients immunized against the polysaccharide antigen, but they show normal survival for up to 26 days when transfused into nonimmune hosts, although these develop humoral antibodies against the bacterial antigen (Shumway, Bockenheuser, Pollock and Neter, 1963). The interpretation is complicated by the uncertainty concerning the question how long the bacterial antigens remain attached to the transfused cells.

The regular survival of blood group incompatible red cells in mice containing antibodies directed against them seems to be a unique characteristic of the species and of the cell type as well. With the exception of certain tumour cells and cartilage grafts no tissues are capable of prolonged survival and growth in spite of an H-2 barrier. The exceptional behaviour of cartilage can be adequately explained by its insensitivity to the homograft reaction, presumably because of the protective influence of the mucopolysaccharide matrix (Stjernswärd, 1964). The situation is more complex with regard to neoplastic tissues; recent studies indicate that non-specific tumours capable of transgressing H-2 barriers have a low concentration of H-2 isoantigens and this makes them resistant to the homograft reaction (E. Möller, 1964). Superimposed on this initial change there seems to be an increased tendency for 'self-enchancement', brought about by the production of humoral antibodies against the tumour cells. The antibodies are unable to kill the resistant neoplastic cells but react with the isoantigens and protect the cells from destruction by immune lymphoid cells (for discussion see Hellström and Möller, 1964).

None of the above mechanisms can account adequately for the survival of incompatible erythrocytes, since these are fully susceptible to the effect of humoral isoantibodies *in vitro*. As pointed out above, this demonstrates the presence of the corresponding H-2 isoantigens on all cells. The target red cells share these antigenic characteristics with other cell types, such as lymphoid cells, which are rapidly rejected in foreign recipients.

The failure of immune elimination in other strains than C57L and C57BL cannot be ascribed to a hypothetical immunological deficiency of the hosts. All strains tested produced humoral antibodies, demonstrable as haemagglutinins, subsequent to the transfusion, but this did not influence the survival of the red cells. The complement level did not seem to be limiting, since 0·5 ml. guinea-pig complement did not increase the efficiency of passively transferred antibodies. The possibility that some host function required for the removal of the antibody-sensitized cells, such as the ability to phagocytose, was overwhelmed and became the limiting factor, was rendered unlikely by the demonstration that a second transfusion of red cells into hosts given incompatible cells and antibodies previously resulted in a rapid elimination of a proportion of the transfused cells. There was no difference between the fraction of cells removed in previously transfused and untreated recipients.

The results may be tentatively ascribed to the existence of two types of antibodies, one blocking the cellular antigens without initiating elimination and the other causing destruction. It would follow that the former type of antibodies would develop earlier than the latter and protect the red cells after transfusion into untreated allogeneic recipients. Furthermore, the blocking antibodies would be more avid *in vivo* than *in vitro*, since the sera which were only partially efficient *in vivo* caused complete lysis of the cells *in vitro*. Although unlikely, this hypothesis cannot be ruled out at present.

Medawar (1959) postulated the existence of two kinds of isoantigens (T- and H-antigens) on mouse cells. H-antigens would be present on erythrocytes, whereas T-antigens would be absent. Only T-antigens would be competent to induce and respond to transplantation immunity mediated by immune lymphoid cells, whereas H-antigens would stimulate the production of humoral antibodies. The possible existence of T-and H-antigens cannot satisfactorily explain the present findings, however, since the red cells, presumed to contain H-antigens only, would be expected to respond to humoral antibodies.

It would appear most likely that the present findings are due to an antibody-induced alteration of the red cells, requiring the participation of some host factors. The change would affect the sensitivity of the erythrocytes to immune reactions (efferent inhibition of the homograft reaction). Evidence for such a change was obtained by the demonstration that passively transferred antibodies did not cause elimination of red cells which had been circulating in incompatible recipients for more than 4–7 days. The changed sensitivity to passively transferred antibodies coincided with the appearance of actively produced antibodies. It may be predicted that the cellular change was caused by an antibody-mediated blocking effect of the antigenic receptors. It seems unlikely, however, that humoral antibodies could achieve this alone, since antibody-coated cells were completely susceptible to immune lysis *in vitro* and also partially eliminated *in vitro*. Interaction between antibodies and some host factors acting by modifying the antigen–antibody complex in such a way that the cells would not be eliminated seems possible. Such a cell would contain no free antigenic determinants and the antigen–antibody–host factor complex would not possess opsonizing ability. Indirect evidence for the participation of host factors is derived from the finding that the same cells were eliminated in C57L recipients but not in

other strains, including A.BY which have the same H-2 complex as C57L. Preliminary studies have indicated that a pronounced change does occur in red cells transfused into antiserum-treated H-2 incompatible recipients (G. Möller, 1964). Such cells become completely resistant to haemolytic isoantibodies *in vivo*, although they had been fully susceptible prior to transfusion and remain susceptible after transfusion in to compatible hosts.

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