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The Immune Response to Heterologous Red Cells in Mice

II. ANTIBODY FORMATION TO RED CELLS FROM SPECIES TAXONOMICALLY RELATED TO SHEEP OR MOUSE

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Summary. An attempt was made to establish the relative immunogenicity of various heterologous erythrocytes in the mouse. The immune response to multi-species red cells was examined by zone centrifugation of the antibodies, and by studying cross-reactivity between the various erythrocytes, time-course of haemagglutinin formation, and finally, antigen dose–antibody response relationships. As compared to sheep erythrocytes, goat erythrocytes were found to have a greatly reduced agglutinability, which merely simulated a reduced immunogenicity. However, erythrocytes from gerbil and hamster proved to be genuinely weaker antigens in the mouse. For alkylating agents, anti-metabolites and anti-mitotic compounds the drug-sensitivity of the immune response to cells of different antigenicity was not altered.

INTRODUCTION

Drugs with the specific ability to induce long-lasting immunological tolerance are much needed. In order to detect such compounds, simple, sensitive and reproducible test-systems have to be developed. In previous publications the immune response to sheep red cells in adult mice was investigated (Dietrich, 1966a) and shown to meet most of the requirements of a screening assay (Dietrich, 1966b). However, compounds displaying very weak immunosuppressive properties might be overlooked under the perhaps rigid conditions of this test, in which, moreover, chemically induced immunological tolerance was of only short duration and required large doses of paralysing antigen (Frisch and Davies, 1966; Dietrich and Dukor, 1967).

Hence, an attempt was made to modify the system by resorting either to conditioned hosts possessing only a reduced number of immunologically competent cells (Dukor and Dietrich, 1967), or else to erythrocytes which are less immunogenic in the mouse.

This communication deals with the production and chemical suppression of antibodies against erythrocytes from species taxonomically related to sheep or mouse. An attempt was made to assess the relative antigenicity based on the number, volume and total surface of the immunizing cells.

MATERIALS AND METHODS

Animals

Colony bred male Swiss albino mice (20–22 g) (Tif 1), gerbils (*Meriones shawi*), golden hamsters and cotton rats (*Sigmodon hispidus*) were obtained from our animal

breeding unit (Tierfarm A.G., Sisseln). Rabbits, White Leghorn chickens, albino rats and guinea-pigs were purchased from local dealers.

Antigens

Whole blood from a single goat, from sheep, guinea-pigs, gerbils, albino rats, hamsters, cotton rats and mice was collected shortly before use in equal parts of a modified Alsever's solution (Bukantz, Rein and Kent, 1946). The cells were washed twice with sterile phosphate-buffered saline (PBS) and re-suspended in PBS to the desired concentration. Mice were immunized by intraperitoneal (i.p.) injection of graded amounts of erythrocytes as mentioned under 'Results'.

Determination of erythrocyte number and size

Fresh erythrocyte stock suspensions, as used for immunization, were diluted in twice filtered formalinized phosphate-buffered albumin saline solution (pH 7·3) (Pruden and Winstead, 1964). Total cell counts were determined by a Coulter electronic particle counter Model B (Coulter Electronics, Hialeah, Florida) (Brecher, Schneiderman and Williams, 1956), using a 100 μ aperture tube. Erythrocyte sizes were measured with the aid of an attached Coulter particle size distribution plotter Model J (Brecher, Jakobek, Schneiderman, Williams and Schmidt, 1962). The relative frequency distributions (Fig. 1) were computed from the size distribution plots of at least five blood samples. Absolute volumes are based on calibration with monosized Paper mulberry pollen. Assuming that the multi-species erythrocytes used in this study had approximately the same shape as human red cells, cell surfaces (S) were computed from corresponding volumes (V) using the formula:

$$S = 1{\cdot}5\left(4{\cdot}83\frac{V}{\sqrt[3]{V}}\right).$$

The correction factor of 1·5 represents the difference between the known mean surface of human erythrocytes and the surface of spheres with the same mean volume (cf. *Documenta Geigy*, 1960). Total cell volume and total cell surface of a given number of cells was calculated by adding up the appropriate values for each size class (Table 1).

Bleeding and testing for humoral antibodies

Mice were bled by orbital puncture. Inactivated individual sera were analysed for haemagglutinins or haemolysins as described earlier (Dietrich, 1966a). The titres are given as reciprocals of the endpoint dilutions expressed as powers to the base 2. Titre 0 was arbitrarily assigned to sera exhibiting no detectable antibody activity at a dilution of 1:2. If necessary, spontaneous agglutination was prevented by adding absorbed homologous serum.

Density gradient centrifugation

This was carried out as described earlier (Dietrich, 1966a).

Drugs

Bayer E 39 soluble (E 39), cyclophosphamide (Endoxan®, Asta) and demecolcine (Colcemid®, CIBA) dissolved in PBS, and 6-mercaptopurine (Waldhof) and 6-thioguanine (Fluka) suspended in PBS were injected subcutaneously on five consecutive days

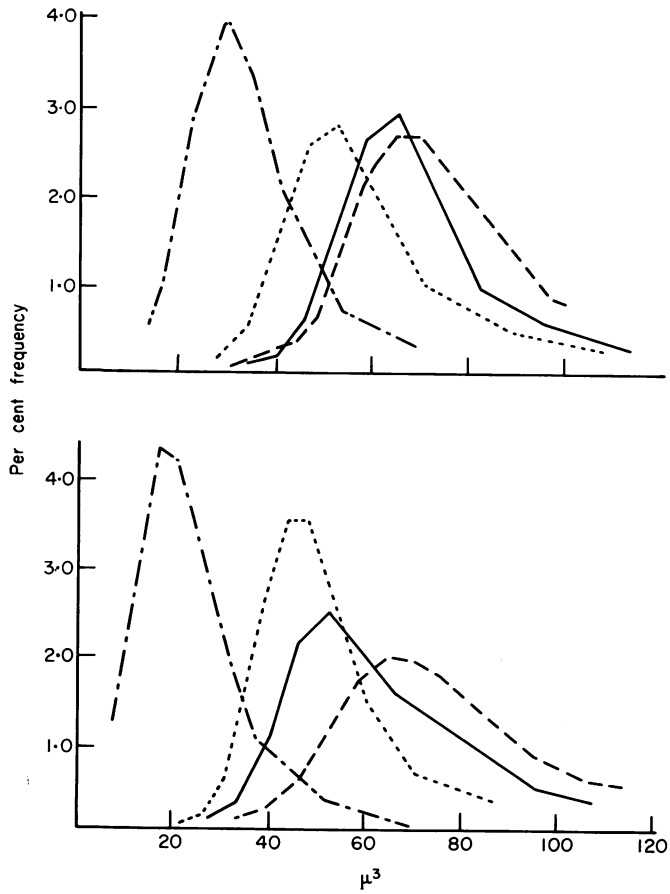

FIG. 1. Frequency distribution plots of erythrocyte volumes from various species. Each distribution curve represents the mean of at least five determinations. (a) — · — ., sheep; . . . ., rat; ——, cotton rat; — — — —, guinea-pig: (b) —· —· —· goat; . . . ., mouse; ——, gerbil; — — — —, hamster.

TABLE 1
TOTAL VOLUME AND SURFACE OF 10^8 ERYTHROCYTES FROM VARIOUS SPECIES

Species	Volume* (μ^3)	Surface* (μ^2)
Goat	3·06	6·98
Sheep	4·07	8·49
Mouse	5·93	10·94
Rat	7·02	12·20
Gerbil	7·39	12·65
Guinea-pig	7·75	13·12
Cotton rat	7·91	13·27
Hamster	8·60	14·00

* $\times 10^9$.

(Dietrich, 1966b). The dosage was adapted to body-weight. The course of drug administration was begun on the same day as the antigen was administered, except in the demecolcine experiments, where drug treatment was started 3 days after the antigen injection. The immunosuppressive effect was evaluated by calculating the suppressive index SI (ratio of average antibody titre of treated animals and average titre obtained from the controls). By plotting SI *versus* dosage (mg/kg body-weight/day) the median effective dosage (ED_{50}) was estimated. By definition, ED_{50} equals the dosage in mg/kg body-weight/day which after five injections causes the SI to drop to 0·5.

Trypsinization of goat erythrocytes

Crystallized trypsin (Sigma) was dissolved in 0·05 N HCl (10 mg/ml) and diluted 1:10, 1:100 and 1:1000 in PBS. One volume of packed, washed goat erythrocytes was added to 4 volumes of enzyme dilution and incubated for either 10 or 60 minutes at 37°, washed again three times with PBS, and re-suspended in this medium to a final concentration of 2·5 per cent. For each enzyme concentration and incubation time, respectively, a four-fold determination of haemagglutinin titres was carried out, using always the same pool of mouse anti-goat erythrocyte serum.

RESULTS

1. THE IMMUNE RESPONSE TO GOAT ERYTHROCYTES

(a) The effect of erythrocyte dosage on antibody formation

The haemagglutinin response to graded doses of goat erythrocytes in groups of seven mice is shown in Fig. 2. Haemagglutination titres against goat cells were low with all

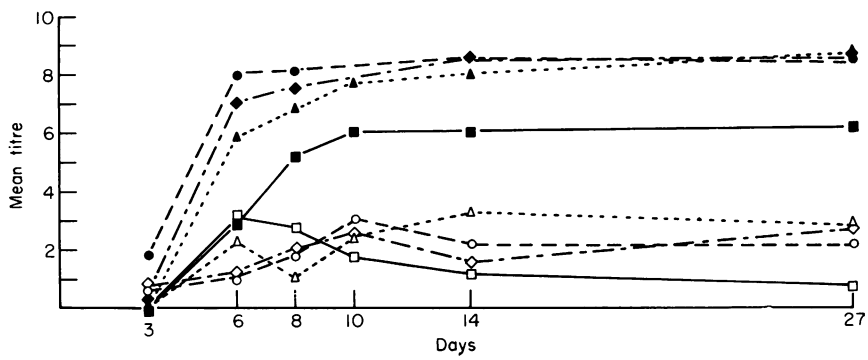

FIG. 2. Haemagglutinin titres of mouse anti-goat erythrocyte sera tested with sheep and goat erythrocytes. Mice were injected with $9{\cdot}5\times10^{6}$ (———), $9{\cdot}5\times10^{7}$ (. . . .), $9{\cdot}5\times10^{8}$ (— • — ·) or $3{\cdot}8\times10^{9}$ (— — —) goat erythrocytes. Open symbols, haemagglutination of goat erythrocytes; closed symbols, haemagglutination of sheep erythrocytes.

doses and were not increased with doses larger than those illustrated. Many sera showed no agglutination or gave atypical patterns; there was no correlation between the doses of antigen and the haemagglutination titres. However, all the anti-goat sera agglutinated sheep cells strongly, giving typical patterns, and titres showed a dose–response relationship. The haemolytic titres of anti-goat sera for goat cells were considerably higher than the agglutinin titres, and were of the same order as those for sheep cells.

(b) Zone centrifugation

Analysis of pooled sera from mice injected with goat erythrocytes is summarized in Fig. 3. Antibody activity located in the neighbourhood of Fraction No. 8 represents 7S antibody, whereas Fractions 13–15 from the bottom of the tubes contain macroglobulins

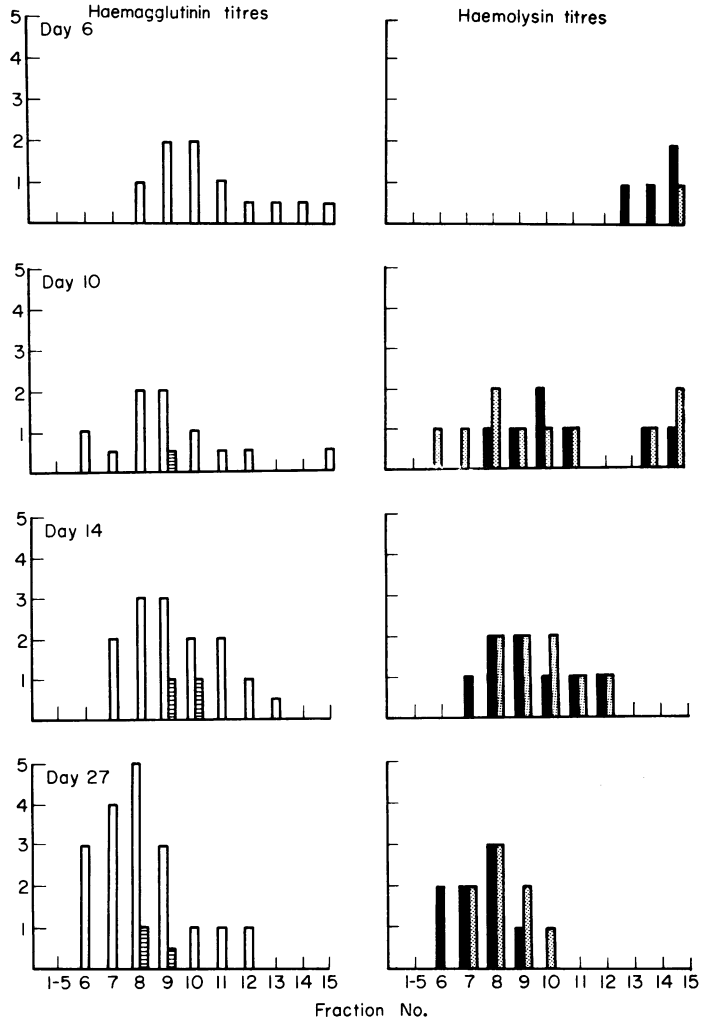

FIG. 3. Density gradient centrifugation of pooled mouse anti-goat erythrocyte sera obtained 6, 10, 14 and 27 days after injection of $3{\cdot}8 \times 10^9$ erythrocytes. Fractions are numbered from top to bottom. Haemagglutinin titres of: fractions tested with sheep erythrocytes (open columns); fractions tested with goat erythrocytes (cross-hatched columns). Haemolysin titres of: fractions tested with sheep erythrocytes (solid columns); fractions tested with goat erythrocytes (stippled columns).

of the 19S type. Each fraction was tested for haemagglutinins and haemolysins against erythrocytes from goat and sheep. On day 6 and day 10 both 19S and 7S haemagglutinins were present. Later in the course of immunization, only 7S haemagglutinating antibodies were found. Again, agglutination of goat erythrocytes was very poor. On the other hand, haemolytic activity against red cells from both species was almost the same in all the

active fractions. Unlike the haemagglutinins, haemolysins on day 6 were restricted to the fractions containing macroglobulins. Moreover, on day 10 a considerable amount of 19S haemolysins was still present, whereas on day 14 and day 27 no haemolysins of the 19S type could be detected any longer.

(c) Cross-reaction with sheep erythrocytes

As mentioned above, goat erythrocytes induced antibodies which cross-reacted with sheep erythrocytes. Similar findings were also obtained using anti-sheep erythrocyte sera from rabbits, chickens and mice which contained haemagglutinins and haemolysins to red cells from both sheep and goat (Table 2). Haemolytic activity against the two

TABLE 2

HAEMAGGLUTINATING AND HAEMOLYSING ACTIVITY OF ANTI-SHEEP ERYTHROCYTE SERA AGAINST SHEEP AND GOAT ERYTHROCYTES

Antisera from:	No. of sera tested	Haemagglutinin titres* against erythrocytes from:		Haemolysin titres* against erythrocytes from:	
		Sheep	Goat	Sheep	Goat
Rabbits	2	8·5	3·0	10·5	12·0
Chickens	12	8·5 ± 1·5	5·2 ± 0·6	8·3 ± 2·0	7·8 ± 2·1
Mice	12	7·5 ± 1·8	2·3 ± 0·9	5·5 ± 0·6	4·0 ± 0·9

* ± standard deviations.

test erythrocytes was of the same order of magnitude. Haemagglutinating activity against goat cells, however, was again greatly reduced.

(d) Effect of trypsin on haemagglutination of goat erythrocytes

Since the foregoing results were strongly suggestive of special properties inherent in the surface of goat erythrocytes, an attempt was made to enhance their agglutinability by pre-treatment with trypsin. As shown in Table 3, trypsinization produced a considerable enhancement of haemagglutinin titres.

TABLE 3

EFFECT OF TRYPSINIZATION ON AGGLUTINABILITY OF GOAT ERYTHROCYTES BY MOUSE ANTISERUM

Time of incubation (minutes)	Haemagglutinin titres*			
	No trypsin	Trypsin		
		1:1000	1:100	1:10
10	1·0	2·3	3·0	3·8
60	1·0	3·0	3·4	3·5

* Average of four determinations

(e) Drug-sensitivity of haemagglutinin formation

The ED_{50} for cyclophosphamide, 6-mercaptopurine and 6-thioguanine was determined in mice immunized with goat erythrocytes and evaluated by testing the sera against erythrocytes from goat and sheep. Drugs were administered to groups of six mice each,

using four different dose levels. As listed in Table 4, the respective ED_{50} values of all three preparations remained almost identical in the two test-systems. Moreover, the median effective dosages compared well with the corresponding figures obtained previously, when sheep erythrocytes were used as immunizing agent (Dietrich, 1966b).

TABLE 4

INHIBITION OF ANTIBODY FORMATION IN MICE IMMUNIZED WITH GOAT ERYTHROCYTES

Substance	Test erythrocytes	ED_{50}
Cyclophosphamide	Goat	11·0
	Sheep	8·0
6-Mercaptopurine	Goat	37·0
	Sheep	40·0
6-Thioguanine	Goat	2·0
	Sheep	3·5

2. THE IMMUNE RESPONSE TO ERYTHROCYTES FROM SPECIES TAXONOMICALLY RELATED TO THE MOUSE

(*a*) *The effect of various amounts of erythrocytes on antibody formation*

The immune response to graded amounts of erythrocytes from rat, cotton rat, guinea-pig, hamster and gerbil is shown in Table 5. Small doses were found to induce a

TABLE 5

IMMUNE RESPONSE OF MICE AGAINST RODENT ERYTHROCYTES

Erythrocytes from:	No. of erythrocytes injected	Haemagglutinin titres*			
		Day 3	Day 6	Days 13–14	Days 27–28
Rat	$6·9\times10^{8}$	1·9±1·2	5·8±1·2	4·6±1·5	3·8±1·2
	$6·9\times10^{7}$	2·1±1·2	5·4±1·5	3·8±1·2	3·6±1·5
	$6·9\times10^{6}$	1·8±1·2	3·5±1·8	3·0±1·6	2·5±1·2
Cotton rat	$5·4\times10^{8}$	5·7±0·6	5·2±0·6	6·3±1·2	6·7±1·3
	$5·4\times10^{7}$	3·6±0·6	4·9±0·3	4·6±1·3	4·9±1·9
	$5·4\times10^{6}$	4·5±0·9	4·3±0·9	4·1±0·9	3·5±0·9
Guinea-pig	$2·8\times10^{8}$	0	4·8±0·7	6·3±1·1	6·5±0·7
	$2·8\times10^{7}$	0	3·4±1·1	5·3±1·4	4·3±1·1
	$2·8\times10^{6}$	0	0·5±1·1	2·6±1·4	2·8±1·1
Hamster	$6·3\times10^{8}$	1·7±1·8	6·6±1·8	5·5±1·8	6·3±1·2
	$6·3\times10^{7}$	0	2·9±2·2	1·0±1·2	2·7±1·6
	$6·3\times10^{6}$	0	0·6±1·5	0	0·8±0·7
Gerbil	$5·6\times10^{8}$	0	6·1±1·2	4·8±1·2	5·4±1·2
	$5·6\times10^{7}$	0	2·4±1·8	1·9±1·5	2·2±1·8
	$5·6\times10^{6}$	0	1·1±1·3	1·6±1·0	1·2±0·9

* ± standard deviations. In most cases twelve sera were tested, except in experiments with guinea-pig erythrocytes, when eight sera were used in each group.

sub-optimal response detectable during the whole period of observation. Larger doses were followed by higher haemagglutinin titres demonstrating a dose–response relationship. About 6 days after immunization haemagglutinins reached a plateau. As judged by latency periods in the various groups and by titres after injection of the lowest cell numbers,

erythrocytes from rat and cotton rat were more immunogenic than those from guinea-pig, hamster and gerbil. The differences in the antigenicity of erythrocytes from these species are further illustrated in Fig. 4, in which the 6-day titres are plotted against the total surface of injected cells. Again, higher doses of erythrocytes from gerbil and hamster were required than from other species in order to produce comparable haemagglutinin titres.

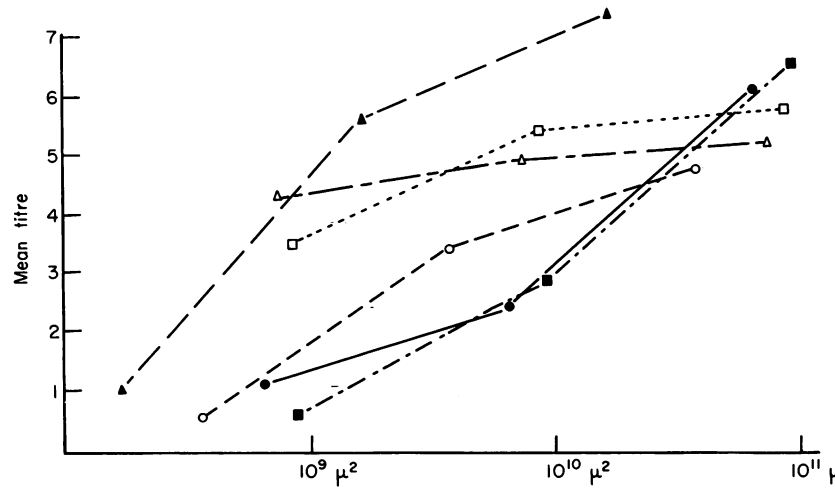

FIG. 4. Haemagglutinin titres 6 days after injection of graded amounts of multi-species red cells. Antigen dose is expressed in terms of total erythrocyte surface injected. Each point represents an average of the serum titrations from eight to twelve mice. ▲, Sheep; □, rat; △, cotton rat; ○, guinea-pig; ■, hamster; ●, gerbil.

TABLE 6

DRUG-SENSITIVITY OF THE IMMUNE RESPONSE TO ERYTHROCYTES FROM SPECIES TAXONOMICALLY RELATED TO THE MOUSE

Immunization with 10^8 erythrocytes from:	ED_{50} of:				
	Cyclophosphamide	E–39	6-Mercaptopurine	6-Thioguanine	Demecolcine
Rat	12·0	12·0	33·0	1·6	1·6
Cotton rat	10·0	9·0	38·0	1·0	3·0
Guinea-pig	14·0	9·0	36·0	1·9	1·0
Hamster*	12·0	11·0	37·0	1·5	1·3
Gerbil	15·0	6·0	23·0	1·6	1·2

* $1{\cdot}6 \times 10^9$ (see text).

(b) Cross-reaction with various erythrocytes

Sera from mice immunized with erythrocytes from the different species were tested against the corresponding erythrocyte panel. Clear-cut cross-reactions could not be detected. Only occasionally did one or two sera show slight agglutination with heterologous erythrocytes. However, five out of nine anti-cotton rat erythrocyte sera exhibited cross-agglutination with rat erythrocytes, some of them showing strong agglutinations in the first serum dilution.

(*c*) *Drug-sensitivity of haemagglutinin formation*

In order to obtain an optimal antibody response, groups of mice were immunized as indicated in Table 6. All suppressive drugs were tested at four different dose levels in groups of ten to twelve mice. The ED_{50} of a given immunosuppressive chemical was found to be nearly the same in the various groups, irrespective of the type of erythrocyte used for immunization. Even where differences were encountered, no systematic correlation with the immunizing antigen could be observed. It would seem that the recorded values lie well within the limits of experimental variation.

DISCUSSION

Erythrocytes of several species were tested for their *immunogenicity* in mice. Significant differences in red-cell-antigenicity were substantiated by a detailed analysis of the erythrocyte dose–antibody response relationship. As seen from cell size frequency distribution plots the erythrocyte populations showed distinct characteristics. It was concluded that antigen dosage should preferably be based on total surface rather than on total cell numbers. Antibody formation against the various types of erythrocytes was found to be characterized by different latency periods after antigen administration, and, even more so, by distinct dose–response patterns.

The results suggest that sheep red cells were more antigenic in the mouse than rodent erythrocytes. Goat erythrocytes were found to cause only a low and inconsistent haemagglutinin response. However, the same anti-goat sera exhibited strong haemolytic activity upon testing with erythrocytes from both goat and sheep, and high agglutinin titres against sheep erythrocytes. Apart from demonstrating the well-known cross-reaction between erythrocytes from goat and sheep (Hyde, 1925), the results also suggest that the reduced anti-goat haemagglutination titres were due to properties inherent in the surface of goat erythrocytes. Indeed, agglutination of these cells was greatly enhanced after pre-treatment with trypsin. Hence, it could be assumed that antigenic sites in the erythrocyte membrane are concealed. Alternatively, haemagglutination may be prevented by steric hindrance due to adjacent protein structures. Pertinent to this are earlier findings indicating the existence of an antigen topography in the membrane of erythrocytes (Bailey and Raffel, 1935; Morton and Pickles, 1947; Gleeson-White, Heard, Mynors and Coombs, 1950; Tomcsik and Scherrer-Gervai, 1962).

Among the rodent erythrocytes tested, those from rat and cotton rat seemed more antigenic than red cells obtained from guinea-pig, hamster and gerbil. Nevertheless, the question might be raised, as to whether these differences were simply due to deficient agglutinability, as was the case for goat erythrocytes. However, the absence of abnormal agglutination patterns, the clear-cut dose-dependency, and the production of fairly high agglutinin titres render this possibility unlikely. It is thus reasonable to assume that genuine variations in red-cell immunogenicity were observed which may reflect the degree of similarity between the antigenic determinants of the donor erythrocytes on the one hand, and the self-constituents of the recipient on the other. Alternatively, it might be argued that our data show some inverse correlation between cell size and antigenicity. Thus, particle size *per se* might influence the immune response by interfering with antigen uptake. However, red cells from the gerbil and rat have similar size distributions, but a clearly differing immunogenicity in the mouse. The same holds true for erythrocytes from the hamster and cotton rat.

The results presented in our study suggest further that *cross-reactivity* between erythrocytes from goat and sheep is not only due to the Forssman antigen, which is shared by the erythrocytes of both species (Forssman, 1930). This was demonstrated by the activity of antibodies induced in Forssman-positive species (mouse and fowl) which are naturally tolerant to the Forssman antigen. In such a system cross-reaction may depend on common (isophilic) antigenic determinant(s) and can be regarded as an expression of the close taxonomic relation between goat and sheep. The extent of cross-reactivity between rodent red cells tested by corresponding mouse antisera was found to be surprisingly low. Only rat erythrocytes showed a weak cross-reaction with anti-cotton rat erythrocyte sera.

In spite of the differences in immunogenicity of some of the erythrocytes tested, antibody formation against all of them showed nearly identical *drug-sensitivity* towards alkylating agents, anti-metabolites and anti-mitotic compounds. It might be assumed, therefore, that all these classes of immunosuppressive chemicals inhibit the proliferation of antibody-producing elements well after the actual stimulation of immunologically competent cells by antigen has taken place. Preliminary evidence indicates, however, that there are other types of immunosuppressive agents which may directly interfere with antigen handling. Antibody formation against rodent erythrocytes with reduced immunogenicity seems to be much more susceptible to inhibition by such compounds than antibody formation against stronger antigens (Dietrich and Dukor, unpublished observations). A model based on multispecies erythrocytes with differential antigenicity in the mouse may also be used for a more detailed analysis of the kinetics of tolerance. Recent experiments from this laboratory suggest that chemical induction of tolerance is directly related to the antigenic properties of the various red cells (Dietrich and Dukor, 1967).

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