ANTI-RED CELL ACTIVITY OF LYMPHOCYTOTOXIC ANTIBODIES: AN *IN VITRO* AND *IN VIVO* EVALUATION*

G. H. VOS, D. ARMSTRONG AND B. G. GROBBELAAR, *Natal Institute of Immunology, Durban*

SUMMARY

The need to obtain non-toxic antilymphocyte sera (ALS) led to the in vitro *and* in vivo *evaluation of its cross-reactivity for red cells. The findings showed that the antibodies coating the erythrocytes* in vitro *are identical with the antibodies that sensitize lymphocytes by the cytotoxicity test. It would appear that the observed similarities are related to sharing of antigenic determinants by both the lymphocytes and red cells. High concentrations of lymphocytotoxic antibodies can be anticipated to sensitize and affect the biological function of many other tissues besides the red cells.*

Intensive immunization schedules were found to produce immune complexes (lymphocyte-antilymphocyte factors) which in turn induced lymphocytosis in place of the anticipated lymphopenia.

Absorption of the immune complex as well as the red cell antibody significantly reduced the toxic properties of ALS but did not improve its immunosuppressive activity.

The short-term exposure of baboons to toxic preparations of baboon ALG was observed to lower their serum complement value. The most likely explanation for this associated activity appears to be complement utilization by avid in vivo *antigen-antibody interaction.*

It is believed that the synthesis of antibodies to intact lymphoid tissue represents a stimulus to multiple antigen determinants. The need to investigate the effectiveness of antibody production to subcellular components of the lymphocyte is indicated before the biological approach to immunosuppressive therapy can be accepted as a safe procedure for man.

In the production of horse antihuman lymphocyte serum (ALS) it is not unusual to find red cell antibodies in combination with antibodies to lymphocytes. This association is in accord with reported observations that red cells and lymphocytes contain common antigenic components.[1] A similar condition of antigen sharing has also been established in studies of the HL-A system where it has been shown that red cell antibodies could almost always be found in preparations of cytotoxic HL-A antisera.[2]

We have noted that horse antihuman lymphocyte preparations often contain extremely high titres of red cell antibodies which, in contrast to any other form of heteroimmune antibody, can be very difficult to remove by repeated absorption with human red cells. More disappointing than the failure to remove the red cell antibody is the observation that repeated absorptions also significantly reduce the antihuman lymphocyte activity. These findings reaffirm that sharing of antigen determinants between the lymphocyte and red cell can be accepted as a normal characteristic. From the studies of Levey and Medawar[3,4] it can be concluded that the lymphocytes also share antigens with thymocytes, spleen cells, kidney cells or lung tissue. The shared antigen configuration may of course not necessarily be expressed to the same degree in different cell lines.

Although it is apparent that antilymphocyte serum has the capacity to exert a direct reaction against tissues other than lymphocytes, several questions seem to be fundamental. Firstly, how does the diversity of antibody reaction arise? Secondly, how does it affect the functional activity of the tissues other than the lymphocytes? This investigation was designed to investigate the *in vitro* and *in vivo* serological and haematological effect of the red cell antibody found in antisera produced against lymphoid cells.

MATERIALS AND METHODS

Preparation of Antilymphocyte Serum

Peripheral human blood lymphocytes were obtained using the method described by Eijsvoogel *et al.*[5] Suspensions of lymphoid cells were injected into horses by the subcutaneous route of administration without adjuvant. The antigen dose varied considerably for the 5 horses used. These details are summarized in Table I and discussed under 'Results'. Baboon (*Papio ursinus*) lymphocytes were obtained by a thoracic duct drainage procedure recently described by Wessels.[6] The total amount of lymphocytes ($8{\cdot}7 \times 10^9$) obtained and injected by subcutaneous route into horse TT (Table I) represents a pooling of lymphoid cells from 6 baboons.

All samples of horse serum were subjected to DEAE Sephadex batchwise separation procedures in order that only the gamma-globulin fractions could be used. To facilitate a realistic serological comparison between the

*Date received: 14 January 1971.

TABLE I. IMMUNIZATION SCHEDULE AND RESULTS

Horse	*No. of subcutaneous injections given*	*No. of donors required for total lymphocytes injected*		*Species of lymphoid cells used*	*Cytotoxicity titre of ALS*	*Immunosuppressive activity*
		Donors	*Lymphocytes* $\times 10^9$			
SC	8	320	95·3	Human	1 024†	Absent
BA	5	200	41·6	Human	512	Present
PR	5	200	64·6	Human	1 024	Absent
VO	3	120	26·8	Human	2 048	Absent
TT	6	6*	8·7	Baboon	512	Absent

*Baboon lymphocytes obtained by thoracic duct drainage.
†Titres are expressed as reciprocals of highest dilution giving positive results.

various samples of horse globulin it was important that the final Sephadex preparations were calibrated to contain approximately the same amount of protein.

Serological Methods

For the determination of red cell antigen-antibody reactions translucent glass tiles were used which could be placed in moist chambers at either 22°C or 37°C, depending on the nature of the test. For comparison of degree of discrimination between positive and negative results, simultaneous standard tests were performed using known positive and negative controls. The sensitization of human or baboon red cells by horse globulin was carried out by incubating equal volumes of a 5% suspension of saline washed cells or enzymated cells (0·5% ficin) with horse globulin at 22°C for 1 hour.

Monospecific rabbit antihuman IgG or rabbit antihorse IgG sera were used for the evaluation of the direct and indirect antiglobulin tests. The antiglobulin sera were absorbed with washed human or baboon red cells to remove species hetero-agglutinating antibodies.

Procedure for the Absorption of Red Cell Antibodies

The absorption of human red cell antibodies from the various horse globulin preparations was always performed on the same day, using the same red cells, to ensure that identical conditions were applied to all samples. Pooled human red cells obtained from four donors (group A_1, group B, group A_1B and group O) were subjected to at least six thorough washings with normal saline. The red cells from each donor were washed separately and pooled at the completion of the saline washings. All horse sera were absorbed with an equal volume of packed washed cells. Unless otherwise stated under 'Results', each specimen of horse globulin was subjected to four absorptions in the following order: two absorptions at +4°C for 1 hour; one absorption at 22°C for 1 hour and one absorption at 37°C for 1 hour. If more than four absorptions were necessary they were carried out at 37°C. In view of the observation that A and B blood group similarities exist between man and baboon it was feasible to use human red cells for the initial absorption of horse antibaboon serum. To ensure absolute removal of antibody activity it was necessary that the final absorption was always performed using pooled baboon red cells.

Lymphocyte Cytotoxicity Test

Cytotoxic titres of ALS were determined by the micromethod recommended by the National Institutes of Health.[7]

Test for Immunosuppressive Activity of ALS

Antilymphocyte serum was tested *in vivo* in vervet monkeys (*Cercopithecus aethiops*) using the procedure of Grobbelaar, *et al.*[8] In brief, full-thickness allografts and control autografts 2 cm in diameter were applied. In untreated monkeys most allografts were rejected in 8 - 10 days and none survived longer than 12 days. ALS was tested by regional administration in order to obtain greater sensitivity, a method introduced by Malek *et al.*[9] The grafts were applied to the inner aspect of the thigh and the ALS given subcutaneously into the leg bearing the graft in a dose of 1 ml serum/kg body-weight/day, starting two days before the graft was applied.

Other Investigations

Quantitative levels of IgG immunoglobulin and serum complement (C'3) were determined by means of radial gel diffusion of the patient's serum in agar gel containing monospecific antibody. Routine haematological studies of packed cell volume, platelet counts and total lymphocyte counts were performed by standard methods as described by Dacie and Lewis.[10]

RESULTS AND DISCUSSION

Antihuman and antibaboon lymphocyte sera were prepared by the subcutaneous administration of lymphocytes into mature horses. The number of injections and amount of lymphocytes given varied for each animal. Table I shows the cytotoxicity titres of the unabsorbed serum samples against peripheral lymphocytes. Horses given high dosage of lymphoid cells did not necessarily produce more potent cytotoxic antibodies than those receiving a low dose.

The ability to lyse lymphoid cells *in vitro* could not be correlated with its *in vivo* immunosuppressive activity. This was evident from the observation that only one of the 6 horses studied showed the capacity to prolong skin-graft survival. The curious fact that the injection of various amounts of lymphoid cells can produce intense cytotoxic antibodies, yet seem to lack the power to prolong graft survival, indicates that the immunosuppressive activity does not seem to reside solely in the capacity of the horse to make cytotoxic antibodies. ALG made against intact lymphocytes inevitably represents a complex mixture of antibodies directed against nuclear and cell-wall material as well as mitochondria and microsomal substances. Moreover, sharing of certain antigen determinants between the lymphoid cell and other tissues can be expected to have obvious disadvantages when antibodies to lymphoid cells are used as immunosuppressive agents. They can, for example, react *in vivo* against tissues other than the lymphoid cell and thereby diminish their selectivity.

Follow-up studies of horses before and after immunization with intact lymphocytes clearly established that the formation of intense anti-red cell activity could always be expected (Table II). Their presence can be observed by a variety of serological procedures including the use of rabbit antihuman globulin serum. The ability of antihuman globulin serum to react with red cells sensitized with horse antihuman ALG is thought to be due to interspecies antigenic similarities between horse and human Fc and Fab fragments, as reported by Allen *et al.*[11]

A comparative evaluation of the red cell antibody observed before and after immunization with lymphocytes showed some remarkable differences with respect to their capacity to be absorbed (Table III). The naturally occurring 'species-specific' horse antihuman red cell factor was always removed by one absorption with packed human red cells but this was seldom observed for the red cell antibody formed by lymphoid cell immunization. Another feature of the lymphocyte-induced red cell antibody is its repeated ability to sensitize the same red cell in spite of many absorptions.

Repeated absorption of preparations of ALG with leucocyte and platelet-free suspension of packed red cells was always observed to alter the lymphocyte cytotoxicity results. Complete absorption of the red cell antibody in

TABLE II. ANTIHUMAN RED CELL ACTIVITY OBSERVED IN VARIOUS HORSE SERUM BEFORE AND AFTER IMMUNIZATION WITH HUMAN LYMPHOCYTES

	Antihuman red cell activity before immunization				*Antihuman red cell activity after immunization*			
Procedures	*SC*	*BA*	*PR*	*VO*	*SC*	*BA*	*PR*	*VO*
Saline agglutination test	32*	32	16	64	2 048	256	1 024	2 048
Agglutination test with enzyme-treated red cells	64	32	32	128	4 096	512	2 048	4 096
Indirect rabbit antihorse globulin test	32	16	32	64	2 048	512	1 024	2 048
Indirect rabbit antihuman globulin test	16	16	16	32	2 048	512	1 024	2 048

TABLE III. COMPARATIVE EVALUATION OF NUMBER OF ABSORPTION S REQUIRED TO REMOVE ANTIHUMAN RED CELL ACTIVITY FROM HORSE 'VO' BEFORE AND AFTER IMMUNIZATION WITH HUMAN LYMPHOCYTES

		Antihuman red cell activity determined by:			
	No. of absorptions	*Saline agglutination test*	*Enzyme-treated red cells*	*Indirect anti-horse globulin test*	*Indirect anti-human globulin test*
Horse VO before immunization	0	64*	128	64	32
	1	0	8	4	4
	2	0	0	0	0
Horse VO after immunization	0	2 048	4 096	2 048	2 048
	1	2 048	2 048	2 048	2 048
	4	512	1 024	1 024	1 024
	8	64	256	256	128
	12	16	32	16	32

*Titres are expressed as reciprocals of highest dilution giving definite positive results.

TABLE IV. ABSORPTION STUDY OF ANTIHUMAN RED CELL ACTIVITY OF HORSE 'SC' BY FRESH AND LYSED PREPARATIONS OF HUMAN RED CELLS AND HUMAN LYMPHOCYTES

Type of antigen used for absorption of antilymphocyte activity present in horse SC		*Horse globulin packed antigen ratio*			
		1:2	*1:4*	*1:8*	*1:12*
Pooled human red cells	Intact preparation	256*	128	64	16
	Stored haemolysed preparation	256	128	32	16
Pooled human lymphocytes	Intact preparation	64	16	2	0
	Stored lysed preparation	4	0	0	0

*The indirect antihuman IgG titre has been expressed as the reciprocal Log_2 of the titre.

fact abolished the cytotoxicity factor, indicating that both the red cells and lymphocytes share antigens with similar structural groupings. That the shared antigen configuration need not necessarily be expressed to the same degree between cell lines was evident in the following study.

When an antilymphocyte serum with potent red cell antibodies is absorbed with increased amounts of red cells and lymphocytes in a comparative test system (Table IV) there appears to be a far greater capacity for lysed lymphocytes to inhibit the red cell antibody than the use of intact or lysed red cells. Similar studies applied to antibodies specifically produced against red cells, in place of lymphocytes, revealed effective inhibition of the red cell antibody with intact or lysed red cells but not with intact or lysed lymphocytes. These findings confirm Brody and Beizer's[1] observation that lymphocyte antibody has a greater avidity for red cells than red cell antibody has for lymphocytes.

It was therefore of interest to evaluate the *in vivo* haematological effect of the red antibody which normally accompanies antilymphocyte activity. Using baboons as the prototype to study the haematological consequence of injected horse antibaboon lymphocyte globulin we observed that unabsorbed antibaboon ALG administered subcutaneously for three consecutive days and again on the sixth day (dosage 25 mg protein/kg) was highly toxic for baboon T1, while the same ALG after absorption with human and baboon red cells appeared to have no effect when given to baboon T11 (Figs. 1 and 2). When baboon T1 received the unabsorbed horse antibaboon ALG with a lymphocytotoxic titre of 1:1 024 and anti-red cell titre of 1:2 048 it became moribund shortly after the fourth injection as a result of generalized anaemia and a potentially dangerous thrombopenia. This effect was not observed in baboon T11 when the same ALG was extensively absorbed with red cells to lower its lymphocytotoxic activity. As a preparation of horse antibaboon ALG it was remarkable to note that it failed to produce the anticipated lymphopenia. The development of a lymphocytosis between the second and twelth day was in fact most obvious in both baboons.

Studies of serum IgG and C'3 levels following the injection of horse antibaboon ALG showed that these parameters can be severely suppressed by the administration of ALG with high red cell titres (Fig. 2). Low concentrations of red cell antibody had no effect on the C'3 value although it was noted to reduce the IgG value slightly. The association of a severely reduced C'3 level with the administration of toxic ALG can be interpreted to mean that the activation of complement need not only be influenced by an allograft rejection mechanism,[12] but that this condition can also be influenced by the passive intro-

duction of complement-fixing antibodies in the form of ALG.

The presence of free antilymphocyte and anti-red cell activity was evident in the serum of baboon T1 which received the unabsorbed antibaboon ALG (Fig. 2), indicating that all the available antigen sites, e.g. lymphocytes, red cells and other tissues sharing lymphocyte antigen configurations must be fully sensitized *in vivo*. Although excess baboon ALG should in this instance have severely affected the survival of lymphocytes, a definite lymphocytosis was observed (Fig. 1), suggesting that that ALG preparation contained a factor with the ability to enhance rather than suppress lymphoid red cell proliferation. It is proposed that the observed lymphoid cell proliferation was induced by the presence of 'lymphocyte-antilymphocyte' complexes in the ALG. Similar responses can be observed *in vitro* when lymphocytes are subjected to preparations of ALG containing antigen-antibody complexes. In such studies it is common to find that ALG-containing complexes can have a marked mitogenic effect on lymphocytes, whereas those lacking immune complexes do not.[13]

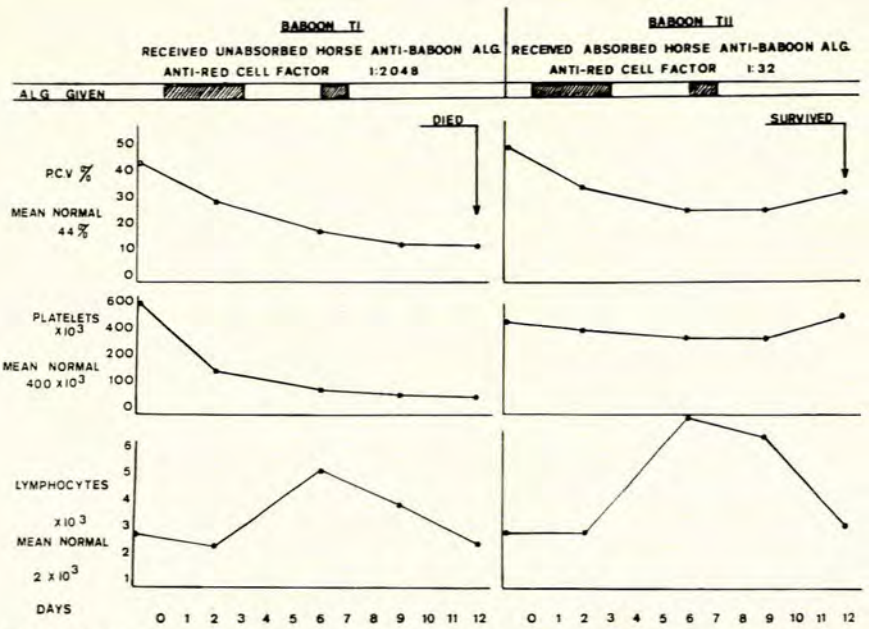

Fig. 1. Haematological alterations seen in baboons following administration of horse antibaboon lymphocyte globulin. Baboon T1 received unabsorbed ALG; antibaboon red cell activity 1/2 048; antibaboon lymphocytotoxicity titre 1/1 024. Baboon T11 received absorbed ALG; antibaboon red cell activity 1/128; antibaboon lymphocytotoxicity titre 1/32.

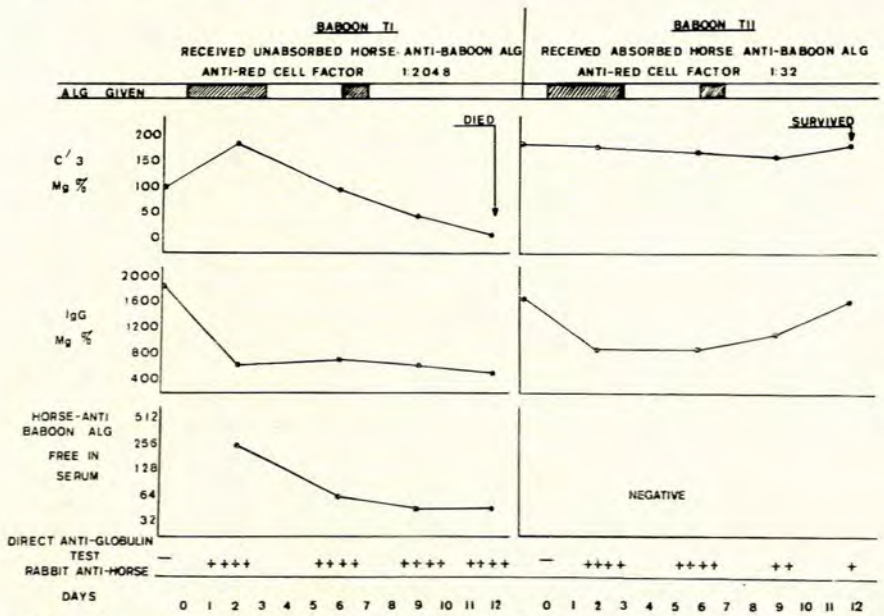

Fig. 2. Serum complement (C'3) and immunoglobulin alteration seen in baboons following the administration of horse antibaboon lymphocyte globulin. Baboon T1 received unabsorbed ALG; antibaboon red cell activity 1/2 048; antibaboon lymphocytotoxicity titre 1/1 024. Baboon T11 received absorbed ALG; antibaboon red cell activity 1/128; antibaboon lymphocytotoxicity titre 1/32.

With the subcutaneous administration of horse antibaboon ALG a firm *in vivo* binding of horse globulin to the red cells of both baboons was confirmed by the direct antiglobulin test using rabbit antihorse globulin serum (Fig. 2). The horse globulin is in this instance the antigenic component of the immunoglobulin with antibaboon red cell specificity. When present on the red cell it can be anticipated to act as an effective adjuvant for the stimulation of antibodies to horse globulin. Baboon T11 produced circulating antibodies to horse globulin after, but not before, the direct antihorse globulin test of the red cells was found to be negative. This sequence of events did not occur in baboon T1 which up to the time of death revealed an excess of cytotoxic horse globulin free in circulation.

The complete absence of lymphocytotoxic antibodies in the serum of baboon T11 should not be interpreted as a positive indication that free horse globulin lacking cytotoxic antibodies cannot be found in the circulation. Until protein purification procedures are absolutely selective a high proportion of non-functional gamma-globulin obtained by group separation methods will inevitably be incorporated with the required antibody. It is therefore a matter of speculation whe-

ther the non-functional horse gamma-globulin or the functional variety (the lymphocytotoxic antibody) should be accepted as the actual stimuli for the synthesis of antihorse globulin in the baboon. The effective separation and use of ALG obtained by specific immuno-absorption procedures would be of interest to evaluate in future studies.

CONCLUSIONS

The need to produce effective, non-toxic antilymphocyte serum is of particular importance in the treatment of organ rejection or auto-immune disease. However, it has been found extremely difficult to produce a highly specific and safe immunosuppressive product. A serious side-effect of antilymphocyte serum is not only its ability to induce thrombocytopenia and anaemia but also its potential to cross-react with many other tissues besides lymphocytes. ALS made against intact preparations of lymphoid cells would therefore appear to contain a complex mixture of antibodies. In the light of this observation it would not be possible to conclude that the immunosuppressive activity of ALG resides solely in its ability to induce lymphopenia.

ALG prepared against a heterogeneous pool of intact lymphocytes represents a mixture of soluble and insoluble antigens in a particularly crude form. Antibodies to such a mixture of antigens will inevitably cross-react with a wide range of other tissues as shown by Levey and Medawar.[3,4] In this condition the prime effect of ALG on lymphocytes cannot be anticipated to be specific since the antibody will also be attached to the surface of other tissues, which in turn alters their functional activity.

Using the haemopoietic cell line as an accessible model to test the side-effects of ALG, in place of inaccessible cell lines (spleen, kidney, lung) we were able to draw the following conclusions from our experiments. When two different potencies of cytotoxic ALG were compared, it was shown that the subcutaneous injection of high-titred antibodies consistently caused severe haemolytic anaemia and thrombocytopenia. This damaging effect on the haemopoietic system could be reduced by repeated absorptions of the toxic ALG with red cells. However, *in vivo* sensitization of the red cells could not be eliminated unless repeated absorptions achieved absolute removal of the red cell antibody. Only at this point did the ALG no longer sensitize the red cells *in vivo*. The absence of red cell antibody activity by repeated absorptions also effectively removed the presence of lymphocyte antibodies. This is consistent with the contention that red cells and lymphocytes share common antigen determinants.

The *in vivo* application of ALG produced by massive dosage of lymphocytes and intense immunization procedures often created a condition of lymphocytosis in place of the anticipated lymphopenia. Complete absorption of the cytotoxic antibody with red cells did not affect the continued observation of lymphocytosis (Fig. 2, baboon T11). On the other hand, absorption of the cytotoxic antibody with a combination of lymphocytes sensitized by antilymphocyte serum (antigen-antibody complexes) consistently abolished the induction of lymphocytosis. However, this *in vitro* manipulation was not found to create a reduction of circulating lymphocytes *in vivo*, which makes it doubtful whether there was any specific immunosuppressive effect present in the first place. The presence of immune complexes in the ALG can of course also play a role in the development of nephrotoxic syndromes by localizing in the renal glomeruli.

The deliberate use of toxic ALG also produced important information with respect to the behaviour of the complement system. This factor was significantly reduced in value as a result of the *in vivo* interaction of ALG with various tissue components possessing shared antigen configuration.

Although antilymphocyte serum can be successfully produced in some horses by repeated injections with heterologous intact lymphocytes, it is still not certain that the synthesized antibody will be specifically reactive for lymphoid tissue because it is known to cross-react with many other tissues. This limitation affects our knowledge of its *in vivo* activity when used as an immunosuppressive reagent.

Investigations are now in progress to isolate and assess the immunological properties of substances obtained from within the lymphocyte.

We wish to thank Drs J. A. M. White, M. A. Rogers and B. C. Wessels for their valuable co-operation in the collection of baboon thoracic duct lymphocytes. This work was supported in part by the South African Medical Research Council (Grant 9/3/12-537).

REFERENCES

1. Brody, J. I. and Beizer, L. H. (1964): J. Lab. Clin. Med., **63**, 819.
2. Rosenfield, R. E., Rubinstein, P., Lalezari, P., Dausset, J. and Van Rood, J. J. (1967): Vox Sang. (Basel), **13**, 461.
3. Levey, R. H. and Medawar, P. B. (1966): Ann. N.Y. Acad. Sci., **129**, 164.
4. *Idem* (1966): Proc. Nat. Acad. Sci. (Wash.), **56**, 1130.
5. Eijsvoogel, V. P., Du Bois, M. J. G. J., Schellekens, P. Th. A. and Van Loghem, J. J. (1969): Transplantation Proceedings, **1**, 408.
6. Wessels, B. C. (1971): J. S. Afr. Vet. Med. Assoc. (in the press).
7. Mittal, K. K., Mickey, M. R., Singal, D. P. and Terasaki, P. I. (1968): Transplantation, **6**, 913.
8. Grobbelaar, B. G., Brain, P. and Rogers, A. J. (1970): Lancet, **1**, 1120.
9. Malek, P., Kolc, J., Haskova, V. and Zastava, V. (1969): Transplantation, **8**, 333.
10. Dacie, J. V. and Lewis, S. M. (1963): *Practical Hematology*. New York: Grune & Stratton.
11. Allen, P. Z., Berger, B. M. and Helms, C. M. (1968): J. Immunol., **101**, 1023.
12. Austen, K. F. and Russell, P. S. (1968): Ann. N.Y. Acad. Sci., **129**, 657.
13. Bloch-Shtacher, N., Hirschhorn, K. and Uhr, J. W. (1968): Clin. Exp. Immunol., **3**, 889.