THE CROSS-REACTIVITY AND TRANSFER OF ANTIBODY IN TRANSPLANTATION IMMUNITY*

By GENSHICHIRO FUJII,‡ M.D., and ROBERT A. NELSON, Jr., M.D.

(From the Laboratories of Microbiology, Howard Hughes Medical Institute, Miami)

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In 1959 (1) a hypothesis was introduced to explain the immunological mechanisms functioning in homograft rejection. The hypothesis was based on the concept of a similar, but not identical, chemical structure of antigens in the tissues of the donor and recipient. After an exchange of tissue between individuals of the same species, antibody (Ab) would be produced with a specificity toward the donor antigens but would react also with antigens in the recipient's tissues. Thus, the primary mechanism involved in homograft immunity would be an expression of an immunochemical cross-reaction of structural groups on molecules possessed by the donor and by the recipient. After production, the Ab would attach to antigens in the recipient tissue whether located on either fixed or mobile cells, provided of course, that the antigens were located on the surface of the cells or were otherwise accessible to the Ab. Under these conditions Ab would not be present free in the serum. As a result of "invasion" of the graft by the sensitized mobile cells of the recipient, it was postulated that the Ab would dissociate or transfer from the cross-reacting antigens on the host's cells (lymphocytes, polymorphonuclear leukocytes, histiocytes, etc.) to the antigens on the donor graft cells responsible for immunization.

This concept of cross-reactivity of antigens shared by individuals of the same species applies equally well to individuals of different species which share chemically similar antigens. One well studied antigen shared by several species is the so called Forssman antigen (2). Many previous studies have indicated a close immunochemical cross-reaction of the antigens isolated from sheep erythrocytes and those isolated from horse kidney and guinea pig kidney, as well as from tissues of other animal species (3–5). From the hypothesis described above, it would be anticipated that the exchange of tissue among heterologous species

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‡ Visiting Investigator, Howard Hughes Medical Institute, Present address: Institute for Infectious Diseases, University of Tokyo, Japan.

possessing Forssman antigens would result in the formation of Ab which would sensitize certain tissues of the recipient. As described in the previous work, it would be expected that this Ab would not appear in the serum under ordinary circumstances. Therefore, special techniques would be necessary to detect and to measure its reactivity. The production of this cross-reacting Ab, from the viewpoint of its combination with host tissue and the possible damaging effects of such combination, such as the fixation of complement (C′), might provide a mechanism for self-damage, or "immunologic suicide", or autoimmune disease. The present experiments, therefore, were designed to reinvestigate the cross-reactivity of Forssman-type antigens from the sheep, horse, and guinea pig as a model for Ab reactions involved in homotransplantation and in certain cases of heterotransplantation wherein the donor and the recipient possess chemically similar antigens.

The initial evidence indicating cross-reacting Ab on recipient cells following skin homografts was obtained by the immune adherence technique (6, 7) which in parallel studies had been shown to detect as little as 0.0008 μg Ab nitrogen per ml of serum. Between 30 and 40 per cent of lymph node cells and leukocytes isolated from homografted guinea pigs were found to react in immune adherence in the presence of guinea pig C′, while only 3 to 8 per cent of cells were reactive when obtained from animals which had received non–cross-reacting antigens such as autografts, heterografts, or injections of vaccinia virus or pollen. Since available data indicated that adhesion reactions with human erythrocytes in the presence of C′ occur only with antigen-antibody complexes, the data were interpreted as evidence that lymph node cells or leukocytes of the homograft recipient were sensitized with Ab *in vivo*. To demonstrate the participation of transfer of Ab in the homograft rejection phenomenon, a model system was employed for technical reasons. This system used guinea pig C′, individual sheep erythrocytes, and hemolytic Ab prepared against individual sheep erythrocytes. The transfer of Ab from the mismatched erythrocyte-Ab complex to the cells of the individual sheep used for immunization was demonstrated to occur at a markedly higher rate than to the cells from another sheep (1). These results provided an indication that the cross-reactivity between the donor and the recipient of the graft and the transfer of Ab from recipient cell-Ab complexes to the donor (graft) cells, which originally served to stimulate Ab formation, are basic factors involved in the homograft rejection phenomenon.

The present study further analyzes this model for transplantation immunity and includes: (*a*) some confirmatory and some new evidence of the cross-reaction, as well as species specificity, of Forssman antigens from guinea pig and horse tissue cells with Ab against sheep erythrocytes; (*b*) methods for measurement of transfer of Ab to intact sheep erythrocytes from cross-reacting complexes with lymph node cells, with lysed lymph cell extract, or with small particles in a methanol extract of certain tissues; and, (*c*) a preliminary study of Ab produced in guinea pigs following injection of sheep erythrocytes.

Materials and Methods

Sheep Erythrocytes.—Blood from several individual sheep was collected into two volumes of modified Alsever's solution (8, p. 149) and refrigerated until used. For hemolytic assays a sample was washed once with 0.0125 M ethylenediaminetetraacetate (EDTA) in saline, and then twice in gelatin-veronal buffer containing Ca^{++} and Mg^{++}. The cells were adjusted to 1×10^8 per ml on the basis of spectrophotometric measurement of oxyhemoglobin equivalent to previous counts of cell number determined on the Coulter electronic counter.

Suspensions of erythrocyte stromata for immunization or for absorption were prepared by lysis with distilled water and alternate washings of the lysed stromata with water and isotonic saline (8, p. 150). As indicated, samples were heated in a boiling water bath at 100°C for 60 minutes and then washed with saline.

Crude methanol extracts of these stromata were prepared as outlined by Rapp (4). One liter of suspension of sheep cells was centrifuged, and the packed cells were dehydrated by addition of five volumes of acetone. The residue was collected in a Buchner funnel, dried by vacuum, and then extracted successively five times with 60 ml of chilled absolute methanol. Each extraction was performed at about 4°C for 12 hours. The pooled extract was clarified by centrifugation at 900 *g* and passed through a Millipore filter. Samples of 100 ml were subjected to vacuum distillation and the dry residue resuspended smoothly in about 80 ml of saline. A few coarse particles were removed by low speed centrifugation. The fine particles were washed twice in saline and resuspended to original volume. Nitrogen (N) analysis by the micro Kjeldahl technique (8, p. 476) showed 0.091 mg N per ml.

Guinea Pig Kidney.—Kidneys were isolated from normal guinea pigs and were ground with sand in a chilled mortar. The paste was washed once with water to remove lysed erythrocytes and twice with saline by centrifugation at 1800 *g* for 30 minutes at 0°C. The washed deposit was dehydrated with acetone and extracted with chilled methanol. This crude methanol extract was concentrated under vacuum and then diluted in saline to yield a suspension of fine white particles. These were centrifuged and washed as outlined above and resuspended in about 15 ml of saline. Nitrogen analysis showed 0.095 mg N per ml.

Horse Kidney.—A normal horse kidney was obtained from a local veterinarian and stored at −35°C. From a 75 gm portion of the kidney, a crude methanol extract was prepared as outlined above. The washed fine particles of the methanol extract were suspended in about 20 ml of saline, yielding a preparation with 0.032 mg N per ml.

Guinea Pig Lymph Cell Suspension.—Axillary and femoral lymph nodes were collected from normal guinea pigs. After removal of adhesive fat tissue, the nodes were teased into 0.0125 M EDTA. The suspension was filtered through glass wool, and washed three times by low speed centrifugation; *i.e.*, 60 *g*, for 20 minutes at 0°C, with gelatin-veronal containing potassium chloride and glucose. Any cells which agglutinated during washings were removed by allowing the suspension to stand for 10 minutes. The concentration of cells was determined by counts in a hemocytometer.

Antibody (Ab).—In most experiments, a pool of 30-day rabbit anti-boiled sheep erythrocyte stromata was used after heating at 56°C for 60 minutes. The concentration of Ab determined by micro Kjeldahl assay of nitrogen uptake on erythrocyte stromata was 1.10 mg Ab N per ml. Rabbit anti-sheep erythrocytes containing 0.46 mg Ab N per ml were obtained on day 30 from a rabbit, No. 1-09, injected with a total of 2.3×10^{11} of cells from sheep 7.

For complement fixation tests, a pool of rabbit anti-sheep cells was prepared from sera collected from six rabbits after 30 days of immunization with a total of 2.3×10^{11} erythrocytes. This serum contained 0.62 mg Ab N per ml. For the preparation of sensitized erythrocytes, a 1/500 dilution of this serum was mixed with an equal volume of a suspension of erythrocytes containing 1×10^9 cells per ml.

A pool of antisera to guinea pig lymph cells was obtained from two rabbits injected with a total of 4.2×10^8 washed normal guinea pig lymph node cells.

Complement (*C'*).—Fresh guinea pig serum collected from our stock of adult males of the Hartley strain was pooled and centrifuged at 46,900 *g* for 45 minutes at 0°C and stored in glass-sealed ampules at about −70°C. This pooled serum was absorbed nine times with packed sheep erythrocytes at 0°C to remove "natural" hemolysins. The absorption was done at 0°C with a 1:20 ratio of washed packed erythrocytes to serum, once for 1 minute, two times for 5 minutes, three times for 10 minutes, and then three times for 15 minutes. The titer of C' in several pools, using 4×10^8 sensitized erythrocytes and incubation at 37°C for 120 minutes, ranged from 260 to 350 50 per cent hemolytic units ($C'H_{50}$) per ml of serum. For use, the serum was diluted to yield 15 ± 1 $C'H_{50}$ per ml. In the experiments on immune adherence, fresh guinea pig serum was absorbed three times with packed human erythrocytes, type 0, at 0°C for 10 minutes each.

Human Erythrocytes.—Blood was collected from normal human beings directly into one volume of modified Alsever's solution. For use as indicator particles in immune adherence, samples were washed as above and adjusted to contain 2×10^8 per ml in gelatin-veronal buffer containing 0.0125 M EDTA.

Diluents.—Veronal buffer (8, p. 149) containing 0.1 per cent gelatin, Ca^{++} (0.00015 M), and Mg^{++} (0.0005 M), designated as gelatin-veronal, was employed for most experiments. As the diluent in experiments with lymph node cells, gelatin-veronal containing 0.2 gm potassium chloride and 1.0 gm glucose per liter, was prepared. Where EDTA was required to block the action of C', veronal buffer with gelatin and 0.0125 M EDTA adjusted to pH 7.3 to 7.5 with sodium hydroxide, designated as EDTA-gelatin-veronal, was employed.

Glassware.—All glassware was cleaned by overnight immersion in concentrated sulfuric acid containing small amounts of sodium nitrate, followed by multiple rinses with tap water, and at least six rinses with distilled water.

RESULTS

Cross-Reactivity of Sheep Erythrocytes with Horse Kidney and with Kidney, Lymph Node Cells, and Platelets from Guinea Pigs.—The ability of varying dilutions of methanol extracts of three of these tissues to react with dilutions of a calibrated antiserum made by immunization of rabbits with boiled sheep erythrocyte stromata was measured by assays for complement fixation, agglutination, and inhibition of hemolysis. Since it was difficult to obtain guinea pig lymph node cells or platelets in quantities adequate for extraction with methanol, direct assays for lysis of sheep erythrocytes were performed with rabbit anti–guinea pig lymph cells and with rabbit anti–guinea pig platelets in order to demonstrate cross-reactivity.

Complement Fixation.—Methanol extract and antistromata were diluted in gelatin-veronal and chilled to 0°C. Five-tenths ml of guinea pig C', containing five $C'H_{50}$, was added to 0.2 ml of twofold dilutions of Ab followed by 0.5 ml of twofold dilutions of methanol extract. After overnight incubation at 0°C, followed by 20 minutes at 37°C, 0.3 ml of sensitized sheep cells (5×10^8 per ml) was added to each tube. After incubation for 60 minutes, the tubes were centrifuged, and the approximate per cent of lysis was measured visually.

Maximal fixation of C' occurred with 0.18, 0.13, and 0.25 μg of N from sheep erythrocytes, horse kidney, and guinea pig kidney, respectively. Approximately two-plus end-points occurred with serum dilutions of 30,000, 17,500, and

20,000 indicating that about 0.037, 0.063, and 0.055 μg Ab N were required to fix four of five $C'H_{50}$ units of guinea pig C′ by antigens isolated respectively from sheep erythrocytes, horse kidney, and guinea pig kidney.

Agglutination.—Methanol extract and antistromata were diluted in buffer containing 0.0125 M EDTA and chilled to about 4°C. Five-tenths ml of twofold dilutions of the extracts was added to 0.5 ml of dilutions of Ab. The tubes were stoppered and placed overnight at 4°C in a rotator turning at about eight RPM. Each extract also was tested with normal rabbit serum. The degree of agglutination was estimated by gently tapping the tubes and noting the size of the aggregates. Controls without Ab were smoothly dispersed and easily distinguished from positive results which were graded from one- to four-plus. Samples from each mixture graded as two-plus were examined microscopically.

Macroscopic aggregation of the fine particles occurred with Ab dilutions of 1/2,400, 1/1,600, and 1/1,600 in mixtures containing extracts of sheep erythrocytes, horse kidney, and guinea pig kidney respectively. No aggregation occurred with 1/25 normal serum. As in the C′ fixation protocol, significantly larger amounts of Ab N were required for aggregation of particles isolated from the two kidney preparations, although the over-all results indicate a considerable degree of cross-reactivity.

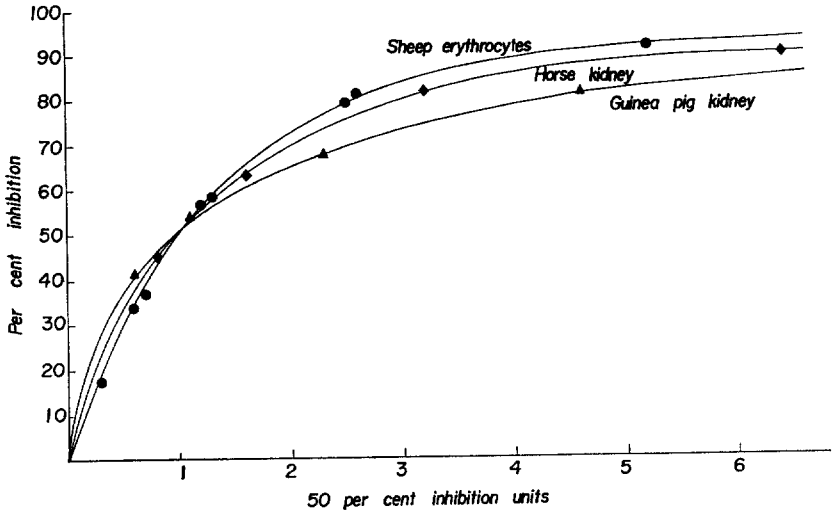
Inhibition of Hemolytic Reactivity.—Six ml of antistromata, containing 0.5 μg Ab N per ml, was mixed with an equal volume of varying dilutions of the three methanol extracts and of varying dilutions of intact sheep cells. After mixing 0°C for 60 minutes on an automatic shaker, the mixtures containing methanol extract were centrifuged at 105,500 *g* for 30 minutes, and the mixtures containing intact erythrocytes were centrifuged at 900 *g* for 15 minutes. The supernatant fluids were separated and stored at 0°C.

The assay for residual Ab was done as follows. Each supernatant fluid was diluted 1/3 with gelatin-veronal, and 1.0, 0.75, 0.5, 0.3, and 0.2 ml, respectively, were added to tubes containing 1.0 ml portions of sheep erythrocytes, 5×10^8 per ml, and amounts of gelatin-veronal adequate to yield a total of 2.0 ml in each mixture. After incubation at 37°C for 15 minutes, 0.5 ml of C′ containing 15 $C'H_{50}$ per ml was added to each tube. After 60 minutes at 37°C, 5.0 ml of cold 0.0125 M EDTA was added, and the tubes were centrifuged. Oxyhemoglobin in the supernatant fluids was measured spectrophotometrically.

The residual units of Ab were calculated from logarithmic plots of the degree of lysis $(y)/1 - y$ *versus* the volume of supernatant fluid, after corrections for spontaneous lysis and C′ color. The percentage of inhibition was calculated from the residual AbH_{50} units as compared with AbH_{50} units determined in a simultaneous assay of 6.0 ml of antiserum mixed with 6.0 ml of gelatin-veronal.

Arithmetic plots were made of per cent inhibition *versus* amount of nitrogen in the methanol extract. From these, the amount of nitrogen producing 50 per cent inhibition was determined. The plot shown in Text-fig. 1 was made comparing per cent inhibition with the various amounts of methanol extract expressed as equivalents of one 50 per cent unit. The results with intact sheep erythrocytes and with the methanol extract of pooled sheep cells were essentially identical and were plotted as a single line. Variations with the methanol extract of horse and guinea pig kidney were similar to those obtained previously

with guinea pig kidney (4). Both kidney preparations showed a slightly higher inhibition with smaller amounts of antigen and moderately lower inhibition with larger amounts of antigen. From these results and from the complement fixation and agglutination assays described above, it may be inferred that the antigens from the three sources were closely related but that the antigen from

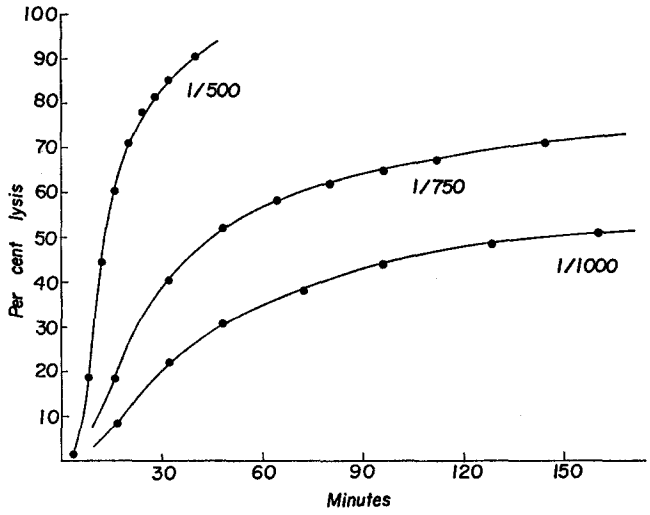


TEXT-FIG. 1. Inhibition of hemolytic reactivity following absorption of 0.5 μg Ab N (rabbit anti-sheep erythrocyte stromata) for 30 minutes at 0°C with methanol extracts of sheep erythrocytes, horse kidney, and guinea pig kidney. One 50 per cent inhibition unit was determined experimentally and found equivalent to 1.9×10^7 intact sheep erythrocytes, 0.13 μg sheep erythrocyte stromata N, 0.05 μg horse kidney N, and 0.07 μg guinea pig kidney N.

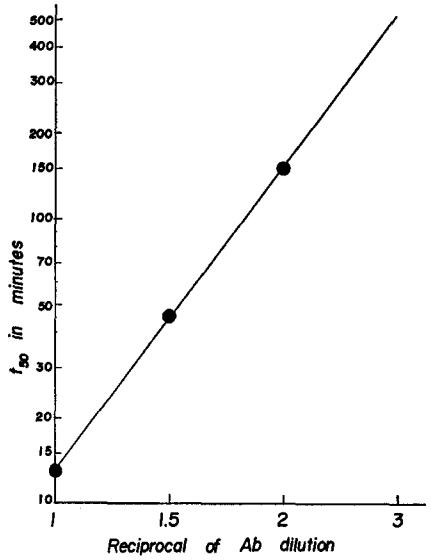
guinea pig kidney was less cross-reactive than was the antigen from horse kidney.

Hemolysis.—Five ml of a 1/500 dilution, 1/750 dilution, and 1/1,000 dilution of an antiserum made by immunization of rabbits with washed cells from guinea pig lymph nodes were added to 125 ml flasks containing 10 ml of sheep erythrocytes, 2×10^8 per ml, and 5 ml of C′, 15 $C'H_{50}$ per ml. The flasks were rocked at 37°C for 160 minutes. One ml samples were withdrawn from the mixtures at varying intervals, and quickly added to 15 ml of cold EDTA-gelatin-veronal. The tubes were allowed to stand in ice for approximately 5 minutes and then were centrifuged. The supernatant fluids were assayed for oxyhemoglobin spectrophotometrically at the wavelength of 415 mμ. Controls for lysis of cells without Ab and for complete lysis were used for the calculation of lysis by the Ab dilutions. Zero time was taken as the time of addition of Ab dilution.

The resulting percentage lysis by the three dilutions of the cross-reacting Ab as a function of time is shown in Text-fig. 2 *a*. In a previous study (9), it had been found that an additional plot of the logarithm of the time required for 50



TEXT-FIG. 2 *a* Hemolytic assay of rabbit anti-guinea pig lymph node cells.



TEXT-FIG. 2 *b*. From values obtained in the kinetic assay a straight line relationship resulted from a logarithmic plot of time required for 50 per cent lysis (t_{50}) *versus* an arithmetic plot of the reciprocal of the relative concentration of rabbit antiserum.

per cent lysis *versus* the reciprocal of the relative Ab concentration resulted in a straight line over a wide range of concentrations for any given antiserum. Such a plot in Text-fig. 2 *b* demonstrates that a straight line also resulted in this cross-reacting system. From this plot it may be calculated that a 1/950

dilution of serum would produce 50 per cent lysis in 120 minutes at 37°C in the presence of about four $C'H_{50}$ per ml of reaction mixture. Similar results were obtained with an antiserum made by injecting rabbits with washed guinea pig platelets. The straight line plot with this cross-reacting Ab yielded a calculated 50 per cent lysis in 120 minutes by a 1/1,460 dilution of Ab. When compared with the hemolytic reactivity of several antisera made by immunization with sheep erythrocytes, these results were suggestive of a reasonably strong hemolytic cross-reaction.

The Transfer of Ab to Sheep Erythrocytes from the Cross-Reacting Ab-Methanol Extract Complex (Species Specificity).—The previous results indicate the formation of an immune complex by anti-sheep erythrocyte stromata and the methanol extracts of sheep erythrocyte, horse kidney, and guinea pig kidney. The next procedures were designed to show the selective transfer of Ab from the cross-reacting antigen to the homologous antigen on the surface of sheep erythrocytes. Subsequent lysis of these erythrocytes as a function of time by C′ only was interpreted as evidence for the quantity of Ab transferred. As will be discussed, the rate of transfer of Ab from a given complex would be expected to vary inversely with the strength of the cross-reaction.

Methanol Extracts of Guinea Pig Kidney.—A sample of anti-sheep erythrocyte stromata was diluted to contain 0.5 μg Ab N per ml. Six-ml aliquots were mixed at 0°C with 6-ml samples of twofold dilutions of guinea pig kidney extract ranging from 4.0 to 0.125 μg N per ml. After mechanical shaking for 60 minutes at 0°C, the mixtures were centrifuged for 30 minutes at 105,500 *g*. The supernatant fluids were removed, and the deposit resuspended smoothly in 10 ml of 0.0125 M EDTA-gelatin-veronal. A total of three such washings was performed, and the sensitized particles then were resuspended in 6.0 ml of EDTA-gelatin-veronal.

The sensitized particles were warmed to 37°C, and 5.5 ml were mixed with 5.5 ml of washed erythrocytes containing 2×10^8 cells per ml in EDTA-gelatin-veronal. These mixtures were shaken at 37°C for 60 minutes (transfer period). Each mixture then was chilled and poured into 50-ml centrifuge tubes containing 5.5 ml of cold gelatin-veronal, and centrifuged at 900 *g* for 10 minutes only in order to separate the erythrocytes from the fine particles of extract which remained in the supernatant fluid. The sheep erythrocytes were resuspended in gelatin-veronal, washed three times, and restandardized to contain 2×10^8 cells per ml. They then were assayed for hemolytic susceptibility in the presence of C′ only.

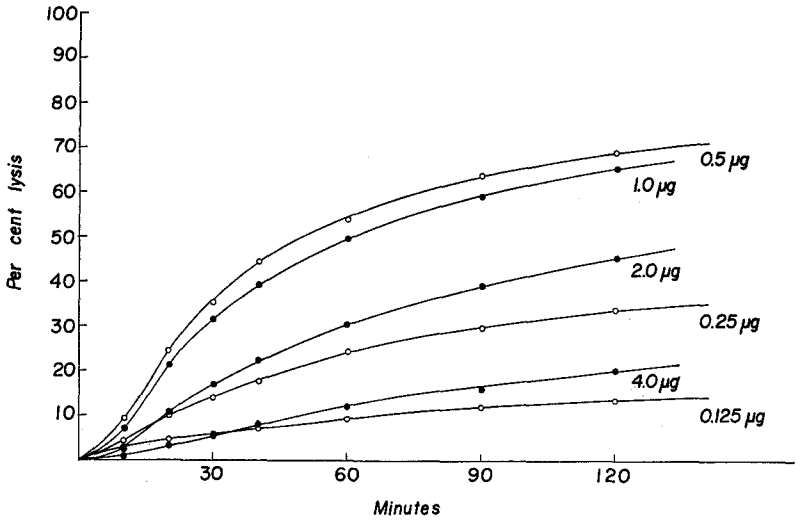
Five ml of each cell suspension was transferred to a 50 ml flask containing 2.5 ml of gelatin-veronal (flasks A through F). Five ml of untreated cells was added to flask G. After shaking at 37°C for 10 minutes, 2.5 ml of a prewarmed 1/25 dilution of C′ was added to each flask (zero time). Flask H served as a control for C′ color. One ml samples were removed at 15, 30, 45, 60, 90, and 120 minutes and added to 15 ml of cold EDTA-gelatin-veronal. After about 5 minutes, these mixtures were centrifuged at 900 *g* for 10 minutes in a refrigerated centrifuge. The supernatant fluids were analyzed spectrophotometrically for oxyhemoglobin. Complete lysis for each flask was determined by removing 1.0 ml and adding it to 15 ml of water.

After correction for spontaneous lysis in flask G, the per cent lysis in each preparation was calculated and plotted as shown in Text-fig. 3.

Significant lysis of the erythrocytes by C′ only occurred with cells which had been incubated at 37°C for 60 minutes (transfer period) in the presence of the

sensitized methanol extracts of guinea pig kidney. The maximal rate of lysis occurred with cells preincubated with the sensitized extract containing 0.5 μg of kidney N.

Methanol Extracts of Horse Kidney and of Sheep Erythrocytes.—Two assays with extracts of horse kidney and of sheep erythrocytes were performed exactly as described above. The methanol extracts were treated for 60 minutes at 0°C, washed three times, and mixed with



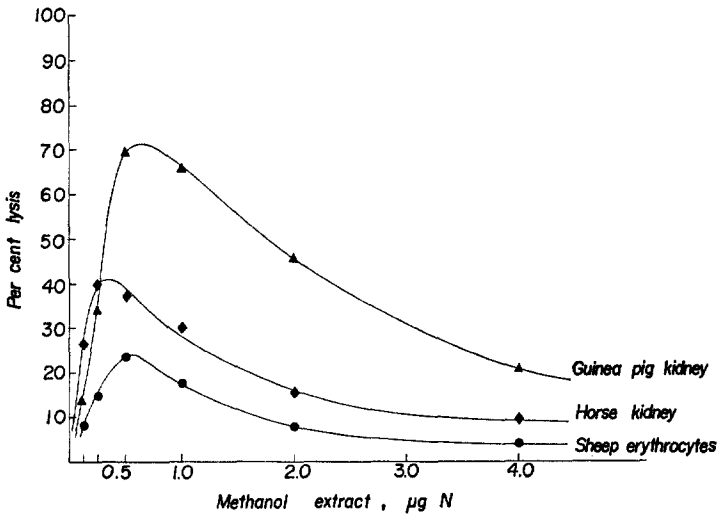
TEXT-FIG. 3. Lysis of intact erythrocytes by C′ as a measure of transfer of Ab from the complex made with 0.5 μg Ab N (anti-boiled sheep erythrocyte stromata) and varying amounts of methanol extract of guinea pig kidney.

sheep erythrocytes for a transfer period of 60 minutes at 37°C. Lysis of the washed erythrocytes was determined as outlined above by sampling from 15 through 120 minutes.

The rate and extent of lysis of sheep erythrocytes were markedly less for cells mixed with sensitized methanol extract of sheep erythrocytes than of horse kidney. A comparison of the results of these transfer experiments was made by plotting the per cent lysis at 120 minutes of each cell suspension against the respective amount of sensitized antigen used in the 60 minute transfer period (Text-fig. 4). It was evident that there was an optimal amount of extract which, when mixed with 0.5 μg Ab N, yielded a maximal transfer of Ab to the sheep erythrocytes. In addition, it may be seen that a distinct gradient existed among the three species in that less transfer occurred in the sequence guinea pig, horse, and sheep. This result was interpreted as further evidence of the degree of cross-reactivity obtained in the assay for inhibition of hemolytic activity (Text-fig. 1). Thus, it may be concluded that Ab can be more readily transferred from a weakly cross-reacting antigen-Ab complex than

from a stronger cross-reacting complex. As will be discussed, this is one basic mechanism involved in individual specificity and in certain cases of species specificity in transplantation immunity.

The Influence of Temperature and Time on Transfer.—In three separate assays, 60-ml portions of methanol extract of guinea pig kidney, containing 0.64μg N per ml, were suspended in EDTA-gelatin-veronal and mixed at 0°C with 60 ml of anti-sheep erythrocyte stromata diluted in EDTA-gelatin-veronal and containing 0.5μg AB N per ml. After 60 minutes a 110 ml



TEXT-FIG. 4. Comparison of lysis of sheep erythrocytes after 120 minutes at 37°C as an expression of transfer of Ab from immune complexes made from 0.5 μg Ab N and varying amounts of methanol extracts of sheep erythrocytes, horse or guinea pig kidney. Maximal lysis was 72 per cent with 0.64 μg N from guinea pig kidney, 41 per cent with 0.33 μg N from horse kidney, and 24 per cent with 0.55 μg N from sheep erythrocytes.

aliquot was chilled and centrifuged and washed three times. The particles were resuspended in 55 ml of gelatin-veronal.

In the first assay, exactly 50-ml portions of the suspension were warmed to 37°C for transfer. In the second assay, the suspensions were brought to 39°C for transfer, and in the third assay the suspensions were kept at 0°C for transfer. Next, 50 ml of erythrocytes, 2×10^8 per ml, was added at the appropriate temperature. Thirty-ml samples were removed from each mixture after 30, 60, and 120 minutes and washed by low speed centrifugation as above. After restandardization and addition of C′ at 37°C, duplicate 1.0 samples were removed at frequent intervals for measurement of oxyhemoglobin as outlined above.

The results of lysis by C′ only are shown in Table I. The time required for 50 per cent lysis of the cells incubated for transfer at 39°C for 30, 60, and 120 minutes was significantly less than those incubated for the same periods at 37°C. Lysis of cells incubated for transfer at 0°C did not reach 50 per cent in-

dicating that dissociation of cross-reacting Ab is markedly depressed at low temperature.

The Transfer of Ab to Sheep Erythrocytes from Washed Sensitized Intact Sheep Erythrocytes (Individual Specificity).—Preliminary studies (1) indicated that a similar transfer of Ab occurred when sensitized sheep erythrocytes were incubated at 37°C with unsensitized cells from another sheep. An attempt was made to establish the optimal time and temperature for transfer.

An individual rabbit antiserum, No. 1-09, 30 day, made by injecting erythrocytes from a single sheep, No. 7, was diluted to contain about 1 μg Ab N per ml. Equal parts of cells from another sheep, No. 8, and the diluted Ab were mixed at 37°C for 60 minutes. This "mismatched" E8-Ab7 complex was washed four times and then lysed completely by addition of C′. EDTA was added, and aliquots of the lysed cells containing 1×10^8 sensitized cells per ml were mixed with equal parts of non-sensitized E7 in 0.0125 M EDTA-gelatin-veronal at 0°C, 25°C, and 37°C. After 30, 60, and 120 minutes of continuous shaking, portions of each suspension were removed, chilled to 0°C, and washed twice with gelatin-veronal. Each batch was restandardized to contain 2×10^8 E7 per ml. Ten ml was pipetted into 50-ml flasks containing 5 ml of prewarmed gelatin-veronal. After 10 minutes, 5 ml of C′ diluted to contain 15 $C'H_{50}$ per ml was added (zero time). Duplicate 1.0 ml samples were removed at 20, 40, and 80 minutes, and added to 15 ml of cold EDTA-gelatin-veronal. The degree of lysis was estimated from oxyhemoglobin determinations of the supernatant fluids following centrifugation at 0°C.

TABLE I

The Effect of Temperature on Transfer of Ab from Methanol Extract of Guinea Pig Kidney to Sheep Erythrocytes as Measured by the Time Required for 50 Per Cent Lysis (t_{50}) of 10^9 Cells

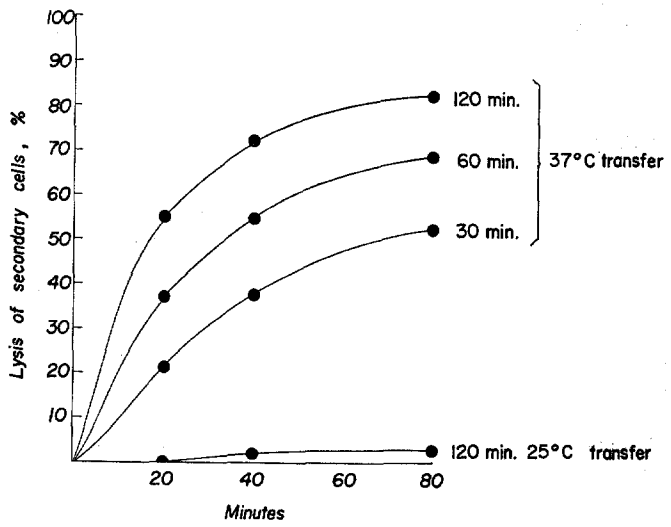
Temperature of transfer*	t_{50} following transfer period of:		
	30 min.	60 min.	120 min.
°C	*min.*	*min.*	*min.*
39	28.5	37.5	44.0
37	33.0	44.8	58.0

* With all samples mixed for transfer at 0°C, lysis was less than 50 per cent even after a lytic period of 150 minutes. The actual per cent lysis at 150 minutes was 3.6 per cent following a 2 hour transfer period and 35.2 per cent following a 24 hour transfer period.

The percentage lysis of cells from sheep No. 7 in the presence of C′ only is shown in Text-fig. 5. An approximate increase of 20 per cent lysis occurred at each time of sampling for the cells incubated for transfer periods of 30, 60, and 120 minutes respectively. While it was not possible to define an "optimal" time for Ab transfer, it may be seen that markedly less transfer occurred at 25°C during 120 minutes, and no lysis occurred either with cells incubated at 25°C for 30 or 60 minutes or with cells incubated at 0°C for 30, 60, or 120 minutes.

Reactions Mediated through Cells from Lymph Nodes.—In previous studies (1)

cells from lymph nodes of homografted guinea pigs showed strong reactivity in immune adherence in the presence of C′ only. The present experiments were designed to test the ability of antiboiled erythrocyte stromata to induce immune adherence, and to demonstrate the transfer of such Ab from washed sensitized cells to intact erythrocytes as a model for events postulated to occur *in vivo* following transplantation of tissues containing antigens which cross-react with the recipient's tissues. In addition, initial attempts were made to



TEXT-FIG. 5. Lysis of sheep erythrocytes, No. 7 (secondary cells), as an expression of transfer of Ab from the complex, erythrocytes 8-antierythrocytes 7, as a function of time and temperature. No detectable transfer occurred during 30 or 60 minutes at 25°C nor during 30, 60, and 120 minutes at 0°C.

demonstrate Ab on the cells of guinea pigs injected with sheep erythrocytes using the techniques and principles developed during the present investigation.

Immune Adherence of Lymph Node Cells.—Cells were isolated from normal guinea pigs, washed and suspended to about 3×10^6 per ml in gelatin-veronal containing potassium chloride and glucose. Dilutions were made of the anti-erythrocyte stromata and of normal rabbit serum, warmed to 37°C, and 0.2 ml portions were added to 0.5 ml of cell suspension. After shaking for 20 minutes, 0.2 ml of a 1/20 dilution of C′, previously absorbed with human erythrocytes, was added. After shaking for 30 minutes, 0.1 ml of human cells, 2×10^8 per ml, was added to each mixture. Controls containing cell suspension only, cell suspension and human erythrocytes, and C′ and human erythrocytes were included. The patterns of the settled erythrocytes were recorded after 60 minutes at 37°C.

Strong immune adherence agglutination patterns occurred in reaction mixtures containing lymph node cells, C′ and rabbit antiserum diluted 1/3200

(Table II). An approximate two-plus pattern was produced with about 0.035 μg Ab N mixed with 1.5×10^6 cells and about 40 $C'IA_{50}$ units of guinea pig C'. Normal rabbit serum showed similar reactivity at a 1/100 dilution. Assuming that equivalent amounts of Ab were required for both two-plus end-points, it may be estimated that the normal serum contained about 17 μg Ab N per ml. The mechanism responsible for the patterns produced by the immune sera was confirmed by darkfield microscopy whereby the specific attachment of sensitized cells to human erythrocytes was easily visualized (Fig. 1).

Specific Inhibition of Immune Adherence.—Three batches of 5 ml of lymph node cells were mixed respectively with 2 ml of 1/100 rabbit anti-sheep erythrocyte stromata, 2 ml of 1/100 rabbit anti–guinea pig lymph node cells, and 2 ml of diluent. After 60 minutes shaking at 0°C the cells were washed three times, and then resuspended to 7.0 in 0.0125 M EDTA-gelatin-veronal. Seven-tenth ml aliquots of each suspension were added to hemagglutination tubes containing 0.5 ml of dilutions of methanol extract of sheep erythrocyte stromata as shown in Table III. After 120 minutes at 37°C, the tubes were centrifuged at low speed, *i.e.* 60 *g*, for 15 minutes to separate the cells from the lighter particles of extract. Each cell deposit was resuspended smoothly in 0.7 ml of buffer and brought to 37°C. These were mixed for 10 minutes with 0.2 ml of 1/20 C', and 0.1 ml of human "indicator" cells was added. The patterns of settled red cells were recorded after an additional 60 minutes at 37°C. Similarly, the supernatant fluids of the original mixtures containing the methanol extract were tested for immune adherence following addition of 0.2 ml of C' and 0.1 ml of human erythrocytes.

TABLE II

Immune Adherence Produced by Guinea Pig Lymph Node Cells Sensitized in vitro with Rabbit Antibody to Boiled Sheep Erythrocyte Stromata

Serum	Immune adherence hemagglutination* with C' and serum diluted:							
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	None
Antistromata‡	4	4	4	4	3	3	2	0
Normal	2	tr§	0	0	0	0	0	0

* The patterns of settled erythrocytes were slightly irregular in all cases due to the presence of lymph cells. Patterns were graded as 0 (no hemagglutination) to 4 (complete hemagglutination).

‡ As counted by darkfield microscopy, the percentages of lymph cells firmly adherent to the indicator erythrocytes were 89 per cent with 1/100 Ab, 31 per cent with 1/3200 Ab, and 7 per cent with no Ab.

§ Tr, trace.

As shown in Table III, the lymph node cells produced strong hemagglutination patterns in the presence of C' following sensitization with rabbit antiserum to either erythrocyte stromata or guinea pig lymph cells. Treatment of the sensitized cells with methanol extract of sheep cells markedly decreased the reactivity of the cells sensitized with anti-sheep erythrocytes but failed to

affect significantly the cells sensitized with antilymph cell antiserum. Insight into the mechanism of inhibition by the cells was derived from the appearance of immune adherence reactivity by the particles in the methanol extract used to treat the sensitized cells. Aliquots from the extract used to treat the unsensitized cells and the cells sensitized with antilymph cells were non-reactive, while aliquots from the extract used to treat the cells sensitized with antistromata produced four-plus patterns. In view of other experiments on transfer of Ab in the present study, it was assumed that Ab had transferred from the cells to the more closely related antigens in the methanol extract.

TABLE III

Immune Adherence Patterns Following Methanol Extract Treatment of Guinea Pig Lymph Node Cells Sensitized with Antibody to Sheep Erythrocyte Stromata and with Antibody to Lymph Node Cells

Rabbit Ab used for sensitization	Material tested in immune adherence	Immune adherence patterns* with material from mixtures of extract:					
		5 μg	1 μg	0.2 μg	0.04 μg	0.008 μg	None
Anti-stromata	Treated cells (deposit)	1	2	2	3	4	4
	Methanol extract (supernate)	4	tr	tr	tr	tr	tr
Anti-lymph cells	Treated cells (deposit)	4	4	4	4	4	4
	Methanol extract (supernate)	1	tr	tr	tr	tr	tr

* A slight disturbance in pattern, recorded as trace (tr), occurred in all mixtures containing methanol extract. Patterns were graded as 0 (no hemagglutination) to 4 (complete hemagglutination).

Transfer of Ab from Sensitized Lymph Node Cells to Sheep Erythrocytes.—Lymph node cells, isolated from normal guinea pigs, were washed and suspended to 3.5×10^7 per ml in special buffer. Mixtures containing 3.5 ml of cells and 3.5 ml respectively of 1/920, 1/4,600, and 1/23,000 dilutions of an individual rabbit antiserum (No. 1-09, 30 day) were mixed for 60 minutes at 37°C. After washing three times in cold diluent and resuspension to the original concentration, 3.0 ml of these sensitized cells was mixed at 37°C for 120 minutes with 0.5 ml of cells from sheep 7, 1×10^8 per ml. As shown in Table IV, 1.0 ml of 1/25 C′ was added to each suspension, followed by incubation at 37°C for a lytic period of 80 minutes. The volume of all mixtures was brought to 7.5 ml by addition of 3.0 ml of cold EDTA. After centrifugation, oxyhemoglobin in the supernatant fluids was measured spectrophotometrically and the percentage of lysis calculated following corrections for spontaneous lysis. Hemolytic assays of the supernatant fluids from the original mixture of lymph cells and Ab also were performed.

As shown in Table IV, 93.2 per cent lysis of erythrocytes occurred in 80 minutes in the mixture containing lymph cells sensitized with approximately 1.5 μg Ab N, and 10.1 per cent in the mixture containing 0.3 μg Ab N. Since the sensitized lymph cells were washed thoroughly and since the wash fluids were found to be hemolytically inactive, the lysis observed herein was attributed to the transfer of Ab from the lymph cell–anti-sheep erythrocyte

complex to the sheep erythrocytes during the 60 minute "transfer period" at 37°C, as well as during the 80 minute "lytic period" at 37°C.

Transfer of Ab from Subcellular Particles of Sensitized Guinea Pig Lymph Node Cells Following Lysis by Water.—Four-ml portions of lymph cells in 0.0125 M EDTA-gelatin-veronal were mixed at 0°C for 60 minutes respectively with 4.0 ml of 1/1,000 and 1/5,000 dilutions of anti-

TABLE IV

Experimental Design and Results: Transfer of Ab from Sensitized Guinea Pig Lymph Node Cells to Sheep Erythrocytes

Tube	A	B	C	D	E	F	G	H
Sensitized lymph node cells, 1.75×10^7/ml, *ml*	3.0	3.0	3.0	3.0	—	—	—	—
Dilution of Ab used for sensitization	1/920	1/4,600	1/23,000	—	—	—	—	—
Erythrocytes, 1×10^8/ml, *ml*	0.5	0.5	0.5	0.5	0.5	0.5	0.5	—
Ab (No. 109), 0.5 μg Ab N/ml, *ml*	—	—	—	—	3.0	—	—	—
Gelatin-veronal, *ml*	—	—	—	—	—	3.0	4.0	3.5
C′ 1/25, *ml*	1.0	1.0	1.0	1.0	1.0	1.0	—	1.0
0.0125 M EDTA, *ml*	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Corrected OD	0.480	0.052	0.003	0	0.515	0	0	0
Lysis, *per cent*	93.2	10.1	0.1	—	—	—	—	—

sheep stromata and with 4.0 ml of diluent only. The sensitized cells were washed three times and then resuspended in 4.0 ml of distilled water. After shaking vigorously for 2.5 hours at 37°C the mixtures were adjusted to isotonicity with 0.3 M NaCl and allowed to stand overnight at 0°C.

Each preparation was centrifuged at 260 *g* for 15 minutes. The cellular fragments in the deposit were resuspended in 4.0 ml of gelatin-veronal. The supernatant fluids were centrifuged at 105,500 *g* for 30 minutes at 0°C. The supernatant fluids were saved, and the deposits were resuspended in 4.0 ml of gelatin-veronal.

Reaction mixtures were prepared containing 0.5 ml of erythrocytes, 1×10^8 per ml, and 3.0 ml of each of the three fractions of the sensitized and lysed lymph cells. After mixing at 37°C for 120 minutes, 1.0 ml of 1/20 C′ was added to each mixture. Hemolysis proceeded during 60 minutes at 37°C after which 3.0 ml of cold EDTA-gelatin-veronal was added to each sample. The percentages of lysed erythrocytes were calculated as usual.

The percentages of lysis of erythrocytes produced by C′ following incubation with the three fractions of the sensitized and lysed lymph cells are shown in Table V. Maximal lysis (98.7 and 28.7 per cent) occurred with the fraction

deposited at 260 *g* with both preparations sensitized with 1/1,000 and 1/5,000 Ab respectively. With the preparations from higher initial Ab, 74.2 and 26.9 per cent lysis resulted in the mixtures containing the fractions in the deposit and supernatant fluid respectively from centrifugation at 105,500 *g*, while 14.2 and 22.5 per cent resulted from similar fractions from the lower initial Ab. A small amount, *i.e.* 2.1 and 2.3 per cent, of lysis occurred in mixtures containing subcellular fragments of non-sensitized lymph cells.

Initial Assays for Ab Produced in Guinea Pigs following Injection with Sheep Erythrocytes.— Aliquots of a suspension of boiled sheep E stromata were injected into two guinea pigs as follows: day 1, 0.5 μg N; day 3, 5 μg N; day 5, 50 μg N; and, day 21, 100 μg N. On day 26

TABLE V

Transfer of Ab from Sensitized and Water Lysed Fractions of Washed Lymph Node Cells to Sheep Erythrocytes as Measured by Per Cent Hemolysis with C' Only

Dilution of Ab used for sensitization	Lysis with fractions of lysed lymph cells from:		
	260 *g*, deposit	105,500 *g*	
		Deposit	Supernate
	per cent	*per cent*	*per cent*
1/1,000	98.7	74.2	26.9
1/5,000	28.7	14.2	22.5
None	2.1	2.3	0.2

(guinea pig 2-14) and on day 32 (guinea pig 2-22) a sample of cardiac blood was collected, and the animals were sacrificed.

A large number of lymph nodes were excised carefully and after washing thoroughly in EDTA-gelatin-veronal, were adjusted to 5×10^7 per ml in gelatin-veronal containing potassium chloride and glucose. Reaction mixtures containing 3.0 ml of lymph cells, 3.0 ml of diluent, and 1.0 ml of sheep erythrocytes, 1×10^8 per ml, were gently shaken at 37°C. After 30, 60, and 120 minutes, 1.75 ml samples were removed and mixed with 0.5 ml of 1/25 C' for an additional 80 minutes at 37°C. The percentage of lysis was measured after addition of 1.5 ml of EDTA-gelatin-veronal.

Another 0.75 ml aliquot of the lymph cells, 5×10^7 per ml, was mixed for 30 minutes at 37°C with 0.75 ml of methanol extract of sheep erythrocytes containing 3.3 μg N per ml. Then, 0.25 ml of erythrocytes was added and the mixtures incubated for 120 minutes at 37°C. Finally, 0.5 ml of 1/25 C' was added, and the percentage of lysis was measured after an additional lytic period of 80 minutes at 37°C.

Assays for hemolytic Ab in the serum of the two guinea pigs were performed in a similar fashion. One ml of dilutions of serum was mixed with 0.5 ml of erythrocytes, 1×10^8 per ml, for 15 minutes at 37°C. The number of Ab H_{50} units per ml was calculated from logarithmic plots of the percentage of lysis *versus* relative Ab concentration. On the basis of these hemolytic assays, 3.0 ml of a dilution of Ab (guinea pig 2-14) containing about seven Ab H_{50} per ml were mixed at 0°C for 60 minutes with 3.0 ml of several dilutions of boiled stromata, and methanol extracts of sheep erythrocyte stromata, guinea pig kidney, and horse kidney. After cen-

trifugation at 144,700 *g* for 30 minutes to remove the particles in the extract, hemolytic assays of the supernatant serum were performed as above.

The mixture of sheep erythrocytes with washed lymph cells isolated from guinea pig 2-14 resulted in 5.6, 15.1, and 52.9 per cent lysis following addition of C′ to samples removed after a transfer time of 30, 60, and 120 minutes, respectively (Table VI). This hemolytic reactivity was almost completely abolished when the lymph cells were first treated with a methanol extract of sheep erythrocytes. Thus, it was tentatively interpreted that injection of the guinea pig with boiled sheep erythrocyte stromata was followed by production

TABLE VI

Injection of Guinea Pigs with Boiled Sheep Erythrocyte Stromata: Assay for Hemolytic Ab in Serum and on Lymph Node Cells by Transfer Technique

Guinea pig No.	Days after injection		Transfer assay			Inhibition of transfer	Serum assay
			Hemolysis after transfer period of:			Lysis after 120 min.	Ab H_{50} units per ml
	Initial	Final	30 min.	60 min.	120 min.		
			per cent	*per cent*	*per cent*	*per cent*	
2-14	26	5	5.6	15.1	52.9	1.9	690
2-22	32	11	1.7	2.5	8.4	0	2050

TABLE VII

Absorption of Hemolytic Reactivity of Serum Ab of Guinea Pig 2-14

Absorbing agent	Inhibition
	per cent
Sheep erythrocyte stromata	56.0
Boiled sheep erythrocyte stromata	47.8
Methanol extract–sheep erythrocytes	0
Methanol extract–guinea pig kidney	0
Methanol extract–horse kidney	0

of Ab of the Forssman-type and that this Ab was combined with cross-reacting antigen on the cells of the guinea pig. In addition, there was a moderately high level of Ab in the serum, *i.e.* 690 Ab H_{50} per ml, which could not be absorbed or inhibited by large amounts of methanol extract of either sheep erythrocytes, guinea pig kidney, or horse kidney (Table VII). Since no inhibition resulted with these Forssman antigens it was assumed that the Ab in the serum was predominately of the "isophile" or non-Forssman-type despite the fact that boiled stromata had been injected.

Results with lymph node cells isolated somewhat later from guinea pig 2-22 were less impressive in that only 8.4 per cent hemolysis occurred after a 120 minute transfer period. In contrast, the serum possessed high hemolytic reactivity; *i.e.* about 2050 Ab H_{50} units per ml. No explanation for the differences is apparent.

DISCUSSION

The well established concept that the affinity of antibody is greater for the antigen used for immunization than for a chemically related cross-reacting antigen is often overlooked when dealing with seemingly complex syndromes *in vivo*. Such would seem to be the case in transplantation immunity wherein only recently has there been evidence presented which implicates the cross-reactivity of donor and recipient antigens (1). With the development of more refined methods there is ample evidence for the cross-reaction of serum and tissue antigens from individuals of different species (10–15). In view of this it might be expected that a high degree of cross-reactivity would occur within the same species and that the production of Ab reactive with one's own tissue would be much more common than ordinarily expected. The present study represents a stage in the development of techniques designed to detect and distinguish the reactivity of Ab with closely related antigens occurring in the same and in different species.

As anticipated from previous work of others, Ab to sheep erythrocytes has been found to cross-react with antigens isolated either as cells or as a methanol extract of guinea pig kidney, lymph node cells, or blood platelets, and of horse kidney. Similar reactivity in complement-fixation, agglutination, and inhibition of hemolysis indicates a close immunochemical relationship between these Forssman-type antigens. The dissociation of Ab from a cross-reacting antigen and its preferential reassociation with a more closely related antigen is the basic mechanism involved in the transfer phenomenon described in the present work. As might be expected, the rate and the total extent of transfer has been found to vary with different antisera, but in these initial studies preferential transfer invariably has occurred when the antigen used for immunization was also used as the secondary antigen mixed with the cross-reacting complex. This was affirmed when rabbit anti-sheep erythrocytes were mixed with a methanol extract of guinea pig or horse kidney. Following extensive washing, the transfer of Ab to sheep erythrocytes was demonstrated by hemolytic assays done in the presence of the cross-reacting antigen-Ab complex and C′.

Two important aspects of the experiments with lymph node cells concern the observation that these cells react strongly in immune adherence when sensitized with Ab to sheep erythrocytes and with C′, and the as yet preliminary evidence that guinea pigs produce Ab which combines with their own lymph cells follow-

ing injection with boiled sheep erythrocyte stromata. The latter is an "auto antibody" in the strictest sense and would appear to provide a convenient model for further investigation into autoimmune diseases and certain types of delayed hypersensitivity wherein serum Ab has not been demonstrated. Immune adherence was inhibited when the sensitized cells first were exposed to a methanol extract of sheep erythrocytes, indicating that dissociation of Ab from the cell surface had occurred. More direct evidence for transfer of Ab was obtained when intact sheep erythrocytes were mixed with the sensitized and washed lymph cells. Lysis of the erythrocytes occurred upon addition of C′. Thus, a well defined model system for immunological phenomena in transplantation immunity has been developed, wherein the production of cross-reacting Ab may be envisaged as responsible for certain types of "cell-bound" or "cytophilic" Ab (16) as well as for the "effector reagent" or so called cell-associated "transfer factor" (17). A further similarity of the latter phenomenon to the present results concerns the fact that the subcellular particles of lymph node cells following treatment with water functioned as complexes of antigen and cross-reacting Ab from which it was possible to show the dissociation or transfer of Ab in the presence of the antigen originally employed for immunization.

These results seem to indicate that individual specificity as ordinarily encountered in homotransplantation is relative and represents a final phase in a syndrome of partial identity as manifested by the cross-reactivity of donor and recipient antigens. Under these circumstances it is not unexpected that little or no Ab activity is found in the serum. In fact, the presence of Ab in the serum of the recipient serves as a strong indication that the donor tissue possessed some antigenic determinants not shared by the recipient. It is evident that Ab either may or may not possess cytotoxic activity, depending upon the location of the antigen in the cell used for immunization and possibly the degree of cross-reaction. In addition, a reasonably simple immunochemical mechanism may be evoked to explain the well known and important role in graft rejection of the mobile mononuclear cells of the recipient. In a strict interpretation of the current results these cells are not "carriers" or "mediators" of the immune reaction but are immunologically specific complexes of cross-reacting antigen with Ab, which serve to transfer and probably to concentrate the Ab on the more closely related antigenic components of the donor cells.

SUMMARY

A close immunochemical relationship of Forssman-type antigens in cells or in a methanol extract of guinea pig kidneys, lymph nodes, and platelets, of horse kidneys, and of sheep erythrocytes was demonstrated by complement-fixation, agglutination, and inhibition of hemolysis. The dissociation of anti-

body from several cross-reacting complexes and re-association with antigens of erythrocytes used for immunization was inferred from quantitative hemolytic assays. This preferential affinity of antibody for the antigen used for immunization is proposed as an immunochemical model for reactions which function in graft rejection phenomena wherein the donor and recipient tissues share cross-reacting antigens.

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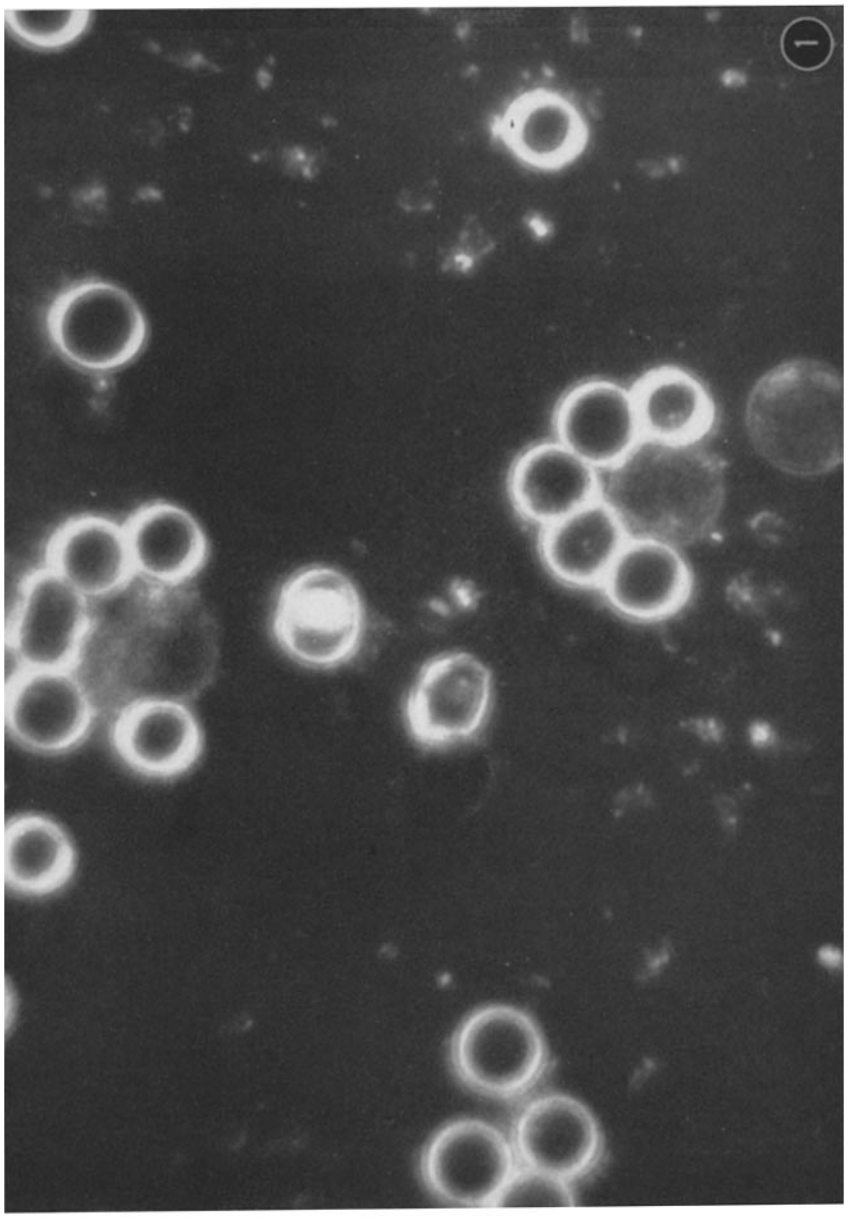
EXPLANATION OF PLATE 89

FIG. 1. Immune adherence of guinea pig lymph node cells cross-reacting with rabbit anti–boiled sheep erythrocyte stromata. Human erythrocytes became firmly attached to the sensitized lymph node cells only in the presence of C′. Approximately × 1000.



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