THE USE OF HETEROLOGOUS ANTILYMPHOCYTE GLOBULIN IN HUMAN RENAL HOMOTRANSPLANTATION*

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IN recent publications (Starzl *et al.*, 1966, 1967; Huntley *et al.*, 1966; Iwasaki *et al.*, 1967), the preparation and testing of horse antidog-lymphocyte globulin (ALG) were described. A preliminary report was also presented on a comparable antihuman globulin which had been used in the treatment of 11 patients who had received renal homotransplants (Starzl *et al.*, 1967). These efforts derived directly from the earlier pioneer work of Woodruff (1960), Woodruff and Anderson (1963, 1964), Waksman, Arbouys and Arnason (1961), Jeejeebhoy (1965), and Levey and Medawar (1966*a*, *b*).

The transfer of heterologous globulin therapy from the laboratory to the clinic has raised two questions which will be discussed in the present report. The first concerns the evidence that a beneficial effect was obtained, a point which requires careful examination since the horse globulin was used only as an adjunct to other commonly employed immunosuppressive agents. Secondly, the potentially toxic properties of the foreign protein have been evaluated from clinical, biochemical, serological, and haematological observations in 24 patients, as well as from a study of homograft biopsies obtained from the first eight kidney recipients approximately four months after transplantation.

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METHODS

Newly operated cases

Case selection and therapy. Twenty consecutive patients received renal homotransplants between 21 June and 12 December, 1966, at the Colorado General and Veterans Administration Hospitals, Denver. In all but two cases, bilateral nephrectomy and splenectomy were carried out at the same time; three of the patients had had thymectomy at an earlier operation. The ages and weights of the recipients ranged from 7 to 47 years and 12 to 75·5 kg. respectively. Horse antihuman-lymphocyte globulin was given each day for five or six days before operation in all but one case, in which the pretreatment was for 35 days. After operation, daily injections were ordinarily given for 10 to 14 days, then every other day for two weeks, twice a week for two months and once a week for one month (Fig. 1). In three instances, treatment was stopped prematurely after $1\frac{3}{4}$ and $2\frac{1}{2}$ months because of toxic reactions to be described below. In all other cases, treatment was discontinued after about four months.

The features of the globulin have been described elsewhere (Iwasaki *et al.*, 1967). It was prepared by ammonium sulphate precipitation of serum obtained from horses previously immunized against human thymus, lymph node, and spleen cells. The injection consisted of γ-globulin, T-equine globulin, and a trace of β-globulin (Fig. 2). The protein content of different batches varied from 4·6 to 9·3 g./100 ml., and the leucoagglutinating titres against human white cells were 1:4,096 to 1:32,768. Administration was intramuscular, in doses of 1 to 5 ml. depending upon the titre of the preparation and the weight of the patient. In order to standardize the dose for patients with varying weights, an arbitrary system was used, based on the conceivably false assumption that the effectiveness was directly related to the leucoagglutinating titre. Thus, 1 ml. of reconstituted globulin with a titre of 1:8,000 was said to contain 8,000 "units". With a titre of 1:16,000, the same volume contained 16,000 units according to this nomenclature. Individual doses were usually about 1,000 units/kg. (range 450 to 2,100 units). During the most intensive period of therapy, early in the postoperative course, the quantity of injected intramuscular protein per week ranged from 14 to 50

mg./kg. During the last month it was 2 to 6 mg./kg. per week. It should be noted that the greatest quantity of horse protein given per week per kilogram was one-third to one-sixtieth of that which was recently shown to cause a high incidence of glomerular lesions in dogs treated with similar antilymphocyte preparations (Iwasaki *et al.*, 1967), and that even this amount was used for only the first 10 to 14 postoperative days.

The development of antibodies against the horse protein was judged by intradermal skin tests, using 0·1 ml. of the same reconstituted ALG given for therapy. Readings were made at 30 minutes, 24 hours, and 48 hours. In addition, haemagglutinin titres against sheep red blood cells were monitored in all patients as an indicator of the response to any Forssman-like antigens present in horse protein (Davidsohn, 1929).

Finally, precipitin titres were followed by two methods of analysis. Initially, a rather crude twofold dilution test was used as described elsewhere (Iwasaki *et al.*, 1967). Later, the stored sera were re-examined by an electroimmunodiffusion technique modified from that described by Laurell (1966) and Merrill, Hartley and Claman (1967). The slide was covered with Difco purified agar containing 5 per cent normal horse serum, and the wells were filled with human serum. Standard curves were constructed by means of double dilution of pooled sera from two patients with known hypersensitivity to horse protein; when plotted on a semilogarithmic graph the length of the precipitates had a straight-line relation to the double dilutions. The precipitation bands of test sera could thus be accurately measured and converted to a unit basis by reading off this standard curve. This provided an extremely sensitive way of following changes in titre in the ALG-treated patients, but only as these related to the unknown precipitin content in the standard serum. Studies are under way to obtain quantitative precipitin titres against known amounts of specific classes of horse immunoglobulins so that absolute nitrogen weights of the precipitins can be measured.

The immune globulin was added to therapy with more commonly used immunosuppressive agents. Azathioprine was given daily from the evening before transplantation and continuing indefinitely thereafter. Prednisone was used as sparingly as possible. It was either withheld until the diagnosis of rejection was made or started in small doses from the day of operation; with

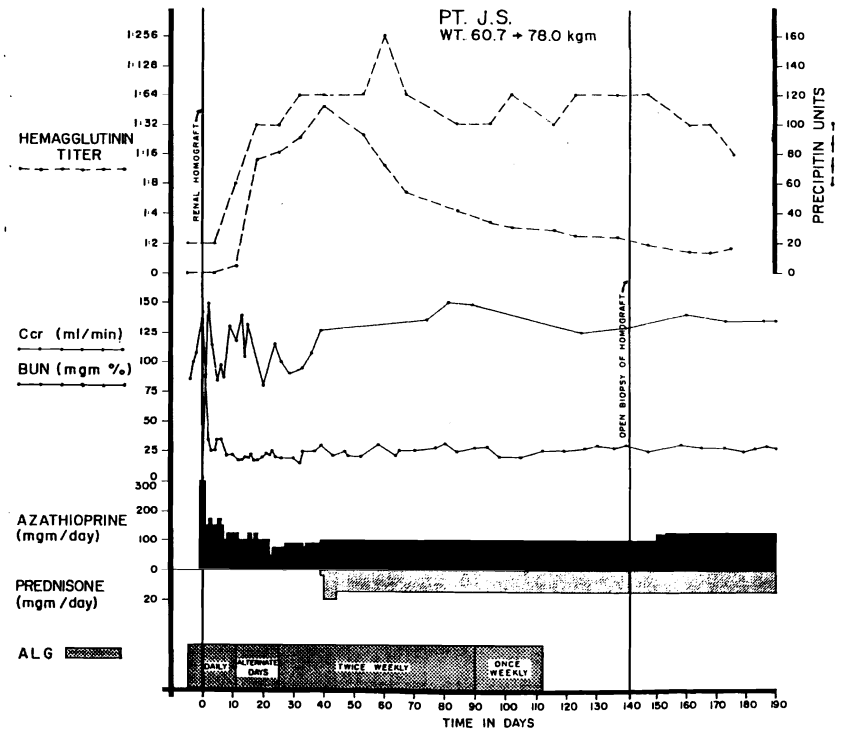

FIG. 1. Course of a patient who received ALG before and after renal homotransplantation. The homograft was provided by a brother. There has never been a rejection. The prednisone was started after 40 days because of the increases in haemagglutinin and precipitin titres. Note the subsequent decline in these measures despite continuation of the globulin injections.

Ccr: creatinine clearance. BUN: blood urea nitrogen.

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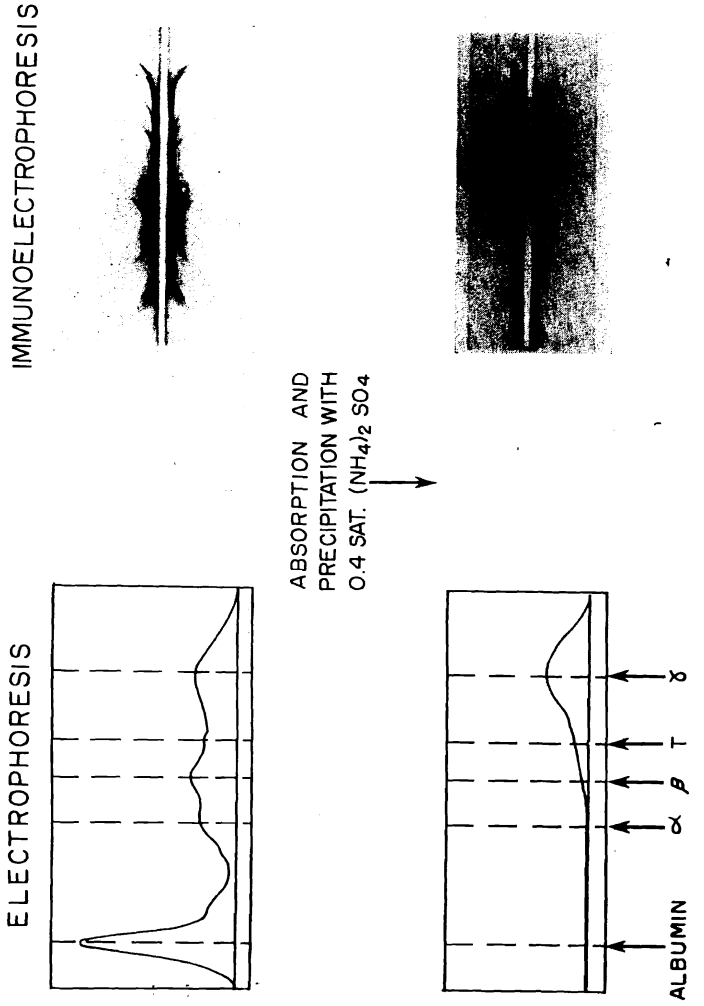

FIG. 2. Electrophoresis and immunoelectrophoresis of absorbed antihuman-lymphocyte serum and the protein obtained from it by two precipitations with 0·4 saturated ammonium sulphate, two dialyses and lyophilization. The final product, which was used clinically, consists almost entirely of IgG. (From Iwasaki *et al.*, 1967. By permission of *Surgery, Gynecology and Obstetrics*, **124**, 1–24.)

the latter therapy the steroid quantities were subsequently increased if rejection was diagnosed.

Patients who had not received steroid therapy and who did not reject transplants during the first two postoperative months were nevertheless eventually placed on daily prednisone doses of 0·31 to 1·37 mg./kg. per day. This was done in some cases because the arbitrary four-month course of ALG therapy was nearing an end. In others, the rise of precipitin or haemagglutinin titres had caused concern about the possibility of impending serum sickness.

In half the cases, 200–400 μg. intravenous actinomycin C and/or local homograft irradiation were used for the treatment of established rejection. The X-ray dose at depth was 150 r. on each of two or three alternate days.

The donors for 19 of the 20 recipients were blood relatives: parents for five, siblings for 13, and a maternal uncle for one. The other patient received a cadaveric homograft. Analysis of donor and recipient lymphocyte antigens was performed by Terasaki and his co-workers (1966) for all pairs. The results were obtained before operation, but did not materially affect donor selection since a choice was available in only three of 20 cases. Furthermore, no potential recipients were denied transplantation during the period of this study on the grounds that a good match could not be found. The donor-recipient pairing was thus not significantly different from that obtainable with random selection. By Terasaki's criteria, an excellent match was obtained in only four cases, an average match in 11, and a poor match in five.

Retrospective control cases. The use of ALG in combination with other immunosuppressive agents complicated evaluation of its effectiveness. Consequently, the influence of ALG upon recovery was studied by comparing the present series with those patients treated in the past at the University of Colorado. Since 19 of the 20 recipients in the ALG series obtained kidneys from blood relatives, the retrospective control cases were limited to consanguineous homotransplantations.

The control patients were divided into three groups of 32, 14, and 25. The first series was compiled between November 1962 and December 1963, the second from January to March 1964, and the third from October 1964 to April 1966. Considerable information was available concerning the lymphocyte antigen conformity

of these earlier patients with their respective donors. Matching was performed retrospectively by Terasaki in 32 donor-recipient combinations in the randomly paired groups 1 and 2, including all but two of the cases which provided data for the 105-day statistical evaluations of function and drug therapy which will subsequently be described. Prospective antigen typing was obtained for all patients in control group 3. In this series a serious effort had been made to find well-matched donors, a trial which was not successful to a statistically significant degree because of the limited number of related volunteers within most families; nevertheless the selectivity which was attempted in group 3 was a distinguishing feature, particularly since a few patients were denied transplantation during this period on the grounds that a reasonable match could not be found. In contrast, it has been pointed out that the donors for the ALG-treated recipients were picked on an essentially random basis and that the latter patients did not enjoy an advantage of better lymphocyte matching by Terasaki's analyses as compared to any of the three control groups.

Division of the retrospective control patients into three consecutively compiled series provided a perspective concerning the effect of increased experience upon mortality. In addition, there were modifications in the techniques of immunosuppression during this time. In the first control group, azathioprine was given for several days before operation. After transplantation, it was continued as the sole therapy until a rejection crisis was diagnosed, at which time large doses of prednisone were added. The second group had similar treatment with azathioprine, but prednisone was used prophylactically in doses of approximately 3 mg./kg. per day from the day before operation. For the third group, prophylactic steroids were also given but in doses of 0·5 to 1 mg./kg. per day. If rejection developed in spite of treatment with azathioprine and steroids, the prednisone dose was raised. In each group, an attempt was made after the first few weeks to reduce the steroid doses as rapidly as possible without precipitating a decline in renal function. As a consequence, the ultimate total prednisone dose in individual cases provided a relatively accurate indication of the difficulty with which rejection was controlled. In all groups, intravenous actinomycin C and/or local homograft irradiation were used irregularly for the treatment of established rejection.

Analysis of clinical data. The influence of ALG therapy was studied by analysing early mortality, the quality of renal excretion obtained from the homografts, and the quantities of azathioprine and prednisone which were necessary to maintain this function. In order to suggest a benefit from the adjuvant use of ALG, it would be necessary to demonstrate a reduced mortality, as well as a diminished need for the other immunosuppressive agents which was not at the sacrifice of renal function.

The relationship between the use of standard immunosuppression and homograft function was compared in the test versus the three control groups in a way which tended to bias the outcome against the ALG series. First, the events in the first 105 postoperative days were documented for all patients who lived this long by calculating for this period the mean value per day of the blood urea nitrogen (BUN), creatinine, creatinine clearance, white blood count, lymphocyte differential, lymphocyte count, azathioprine dose, and prednisone dose. When these data were pooled in each group for the entire period of 105 days, it could be seen whether the overall function in the ALG cases was as good as in previous series, whether this function had been achieved with greater or smaller doses of standard immunosuppressive agents, and whether there were consistent differences in the white cell differential and total counts in the various groups. Each of the first ten patients treated with ALG contributed to this analysis since there were no deaths. In contrast, nine, two, and three, respectively, of the 32, 14 and 25 patients in control groups 1, 2 and 3 were excluded because of early death, a number of highly unfavourable cases being thereby eliminated.

The foregoing analysis provides a composite view of the total early course, but the pooled data would not identify postoperative changes of either deterioration or improvement in function with the passage of time, a point of particular importance since globulin therapy was attenuated and stopped during the first four post-transplant months. Therefore, for the first eight patients treated with ALG the average weekly BUN levels and creatinine clearances for the first 5½ months after transplantation were compared with those from the 23, 11, and 22 patients in control groups 1–3 who lived this long. Graphs of results plotted on a week to week basis permitted a more concise analysis of trends in both the test and control series.

Histopathological studies. The first eight patients in the ALG series had biopsies of their homografts taken from 108 to 145 days after homotransplantation. The last injection of horse protein had been from 1 to 30 days previously in seven of the cases, and 56 days before in the eighth. At the time of biopsy, all of these recipients had excellent renal function. The mean BUN for the group was $26 \cdot 7 \pm 5 \cdot 5$ (S.D.) mg./100 ml., plasma creatinine $0 \cdot 92 \pm 0 \cdot 15$ (S.D.) mg./100 ml., creatinine clearance $97 \cdot 4 \pm 21 \cdot 2$ (S.D.) ml./min., inulin clearance $60 \cdot 9 \pm 12 \cdot 6$ (S.D.) ml./min., and para-aminohippuric acid (PAH) clearance $348 \cdot 9 \pm 65 \cdot 5$ (S.D.) ml./min. The filtration fraction was $17 \cdot 9 \pm 3 \cdot 3$ (S.D.) per cent. Urine protein concentration was $5 \cdot 4 \pm 3 \cdot 6$ (S.D.) mg./100 ml. Dr. David A. Ogden, who performed these clearances, also studied all eight donors at the same time. The pooled function in the recipients was not significantly different from that in their donors.

Large wedge biopsies were removed under local anaesthesia, and divided into four portions. One part was fixed in 10 per cent buffered formalin, embedded in paraffin wax, serially sectioned and stained with haematoxylin and eosin, periodic-acid Schiff and Weigert's elastic-tissue stain. Another part of the biopsy was immediately cut into small cubes, fixed in Palade's buffered osmium tetroxide and embedded in Epon 812. Sections $0 \cdot 5\ \mu$ in thickness cut from these blocks were stained with Azure II and examined by light microscopy. Very thin sections for electron microscopy were stained with lead hydroxide, uranyl acetate or a combination of both, and examined in a Siemens Elmiskop IA. The third piece of tissue was snap-frozen and examined by Dr. G. A. Andres of the Istituto Patologia Medica, Università di Roma, Rome, and by Drs. K. C. Hsu and B. C. Seegal of the Department of Microbiology, Columbia University, New York. Sections were cut in a cryostat and the unfixed tissue stained with fluorescein-labelled antisera to human immunoglobulin G (IgG), immunoglobulin M (IgM), complement (β_{1A} and β_{1C} globulins), fibrinogen and albumin, and to horse globulin, rabbit globulin and Type 12 streptococcus. The anti-IgG sera was kindly supplied by Dr. A. J. L. Strauss (Strauss *et al.*, 1964). Antisera to β_{1A} and β_{1C} were obtained through the courtesy of Dr. H. G. Kunkel (Lachmann *et al.*, 1962). The fourth part of each biopsy was used by Drs. Andres, Hsu and Seegal for ferritin-labelled antibody studies which are not yet complete and will be reported elsewhere.

In addition, autopsy tissue became available from the 14th patient in the series who died 42 days after homotransplantation of a fraternal kidney, and eight days after placement of an additional cadaveric homograft.

Late cases

Four patients had received homografts from 5 to 11 months previously, two from cadavers and two from their mothers. In each case, deterioration of renal function occurred when the prednisone dose was reduced below $0 \cdot 5$ to $1 \cdot 7$ mg./kg. per day. In addition, three of the four patients had developed serious infectious complications.

In all four cases, a four-month course of ALG therapy was started, similar to that described earlier for newly operated cases. After a few days, a drastic progressive attenuation of the prednisone dose was begun which reached a low of $0 \cdot 23$ to $0 \cdot 3$ mg./kg. per day in three patients; azathioprine was continued. All immunosuppressive therapy was eventually stopped for the fourth patient.

RESULTS

Newly operated cases

Mortality. The patients received transplants from five weeks to seven months ago. The operations were almost six to more than seven months ago in the first eight cases, $2\frac{1}{3}$ to $4\frac{1}{2}$ months ago in the next eight, and approximately $1\frac{1}{2}$ months ago in the last four.

There has been one death (Fig. 3). A 43-year-old man died 42 days after bilateral nephrectomy, splenectomy, and receipt of a homograft from his brother, and eight days after a secondary cadaveric homotransplantation. The first kidney was seriously damaged by a postoperative wound haemorrhage originating from a venous infarct of the ureter. The kidney then passed through a 25-day period of anuria before resuming subnormal function. The second homograft never excreted urine. The patient died of pneumonitis. At autopsy the first transplant weighed 320 g. and the second weighed 160 g.

Microscopically, almost all the glomeruli in the first renal homograft contained crescents formed by proliferating capsular epithelium. There was also focal thickening of the glomerular capillary basement membranes and an increase of mesangial

matrix. Several tufts were hypercellular because of mesangial and endothelial cell hyperplasia. The tubules were lined by flattened atrophic epithelium and there was general interstitial oedema and fibrosis. Several interlobular and arcuate arteries were slightly narrowed by fibrous intimal thickening. There were a few infiltrating mononuclear cells in the interstitium and in the

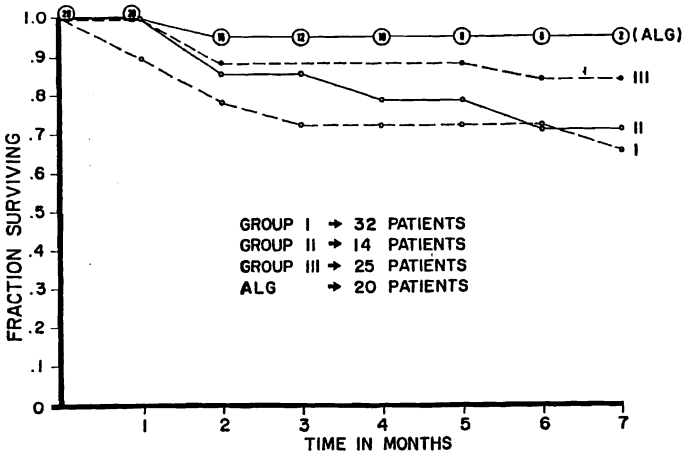

FIG. 3. Mortality in patients treated with adjuvant ALG compared to that in three preceding series of consanguineous transplantations carried out in Denver. The numbers in the ALG series refer to the patients at risk at the indicated times.

peritubular capillaries. It was thought that the glomerular changes in this transplant were the result of transmission of glomerulonephritis from the host; the original clinical diagnosis of rapidly progressive acute glomerulonephritis had been confirmed by pathological study of the patient's diseased kidneys. The only evidence of a homograft reaction was the mild cellular infiltration and minor vascular changes.

In the second renal homograft there was evidence of healing acute tubular necrosis with accumulation of calcium oxalate crystals, but no evidence of a homograft reaction.

Survival. Nineteen of the 20 patients are alive. The early fate of these recipients was compared to that during a comparable time

in control groups 1–3 (Fig. 3). The ALG-treated patients had the lowest early mortality. Furthermore, there have been no delayed deaths in the first 12 consecutive cases after an observation period of three to seven months. The immediate impression was that for

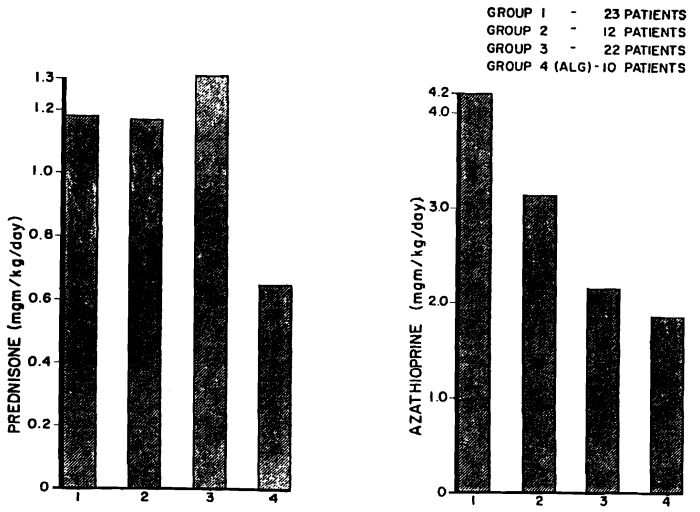

FIG. 4. Average daily doses of azathioprine and prednisone for the first 15 postoperative weeks in three preceding series of consanguineous homotransplantations done in Denver, compared to the doses used for the first ten consecutive patients treated with adjuvant ALG. Since inclusion in the study was contingent upon survival for this period, nine, two and three other patients from the retrospective control series were eliminated by their early death; such a correction was unnecessary in the ALG cases. Note that the ALG-treated patients received reduced quantities of the "standard" immunosuppressive agents.

reasons requiring further clarification the loss rate in this group was cut during both the acute and chronic phases of convalescence.

Immunosuppression and function. During the time when control groups 1–3 were compiled, there was a progressive tendency to use smaller doses of azathioprine (Fig. 4) in an effort to prevent the bone marrow depression and sepsis which were responsible for most of the early deaths. This trend continued into the first 105-day period of the ALG series, during which the azathioprine doses

were smaller than in any preceding group (Fig. 4). The linear downward change was statistically significant ($P<0{\cdot}005$).

In contrast, the average steroid doses during the first 105 days of recovery were almost identical in the first two control groups

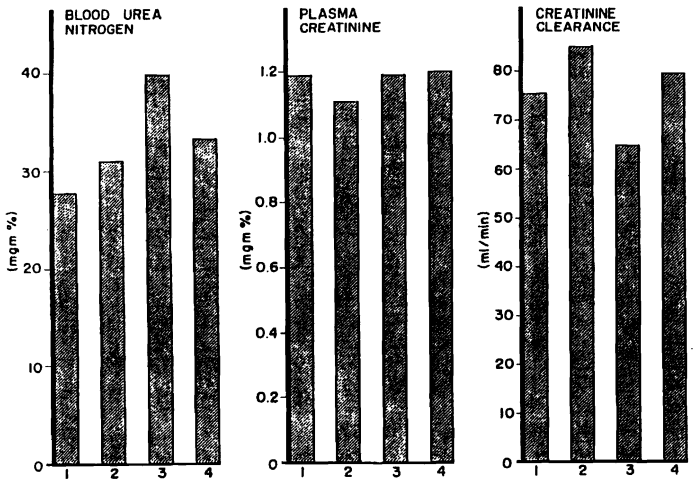

FIG. 5. Average weekly renal function for the first 15 post-transplant weeks in the same retrospective control and test cases shown in Fig. 4. The BUN and creatinine values were not significantly different in any of the series, but the creatinine clearances in group 3 were depressed to a statistically significant degree ($0{\cdot}025>P>0{\cdot}01$). See text for details.

and they were increased in the third (Fig. 4). The ALG series was markedly different. The average daily dose was 0·65 mg./kg., a statistically significant reduction ($P<0{\cdot}005$) compared to 1·18, 1·19 and 1·31 mg./kg. in the three control series.

The advantage of treating the patients in the ALG series with smaller quantities of standard immunosuppressive drugs was not achieved at the price of a reduction in quality of homograft function. The average BUN and plasma creatinine in the first ten ALG-treated patients for the first 105 postoperative days were not significantly different to the levels in the successive control groups, and the creatinine clearances were better than in all except retrospective control group 2 (Fig. 5). In interpreting this finding, it is

important to re-emphasize that inclusion in the statistical evaluation was contingent upon survival for the study period, a requirement which was met by each of the first ten ALG-treated patients.

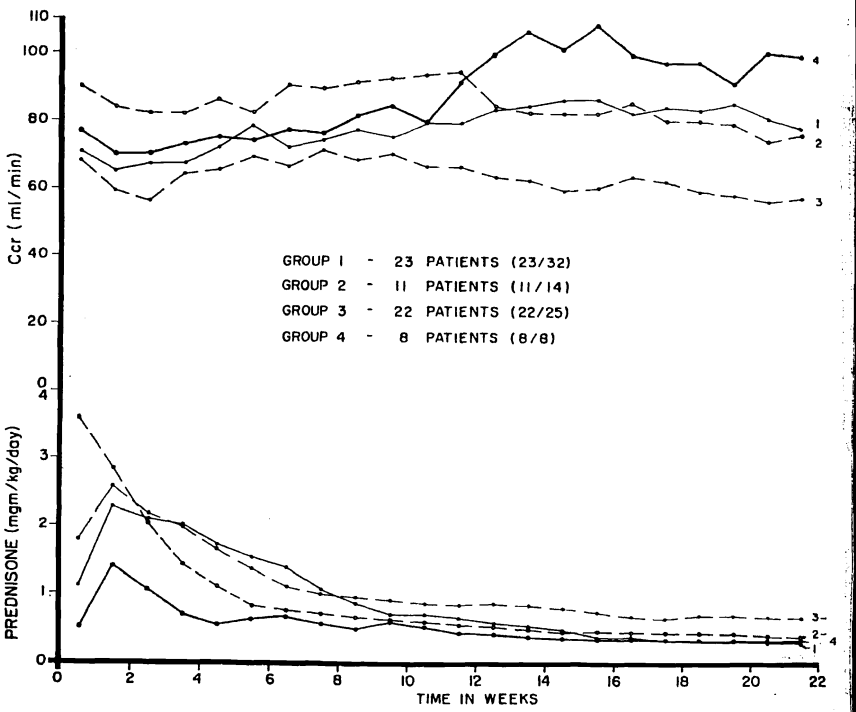

FIG. 6. Relation of average prednisone doses to creatinine clearances (Ccr) during the first 22 weeks for the first eight ALG-treated patients, compared to comparable data in the three retrospective control series. Note that the patients in the ALG group received the smallest quantities of steroids, and that they ultimately had the highest average clearances.

In contrast, 14 unsuccessfully treated control patients who generally had poorer renal function and higher drug doses than the other members of their respective series were excluded. The favourable showing of the ALG group was in spite of this bias.

Further analysis of the weekly renal function of the first eight ALG-treated patients during the first 5½ postoperative months provided a more dynamic view of this test series, as compared to

the control cases in which there was survival of at least this interval. There was little difference in any of the groups for the first two months. After this, the creatinine clearances decreased in control groups 2 and 3. These measures increased in control group 1, but at only half the rate of an increase in the ALG-treated patients (Fig. 6). The late linear trend in creatinine clearance in the

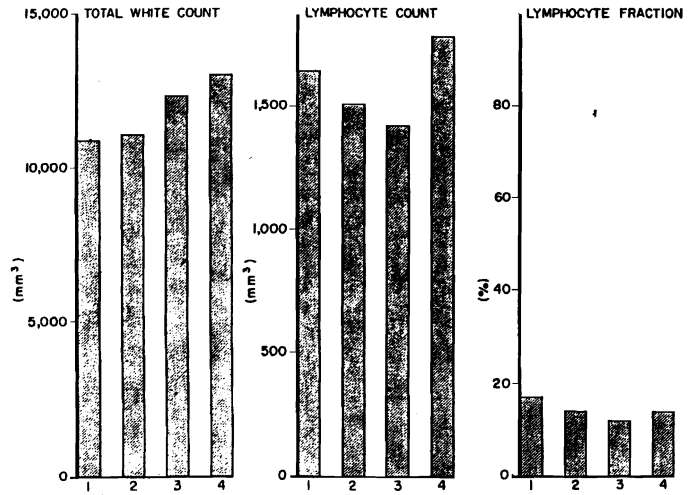

FIG. 7. Average total white cell counts, lymphocyte counts, and lymphocyte differential counts in the same series shown in Figs. 4 and 5, during the first 15 postoperative weeks. See text for discussion.

ALG series was significantly different ($P < 0{\cdot}005$) from that in any of the preceding controls. These findings indicate that deterioration of function did not occur during and after the attenuation and final discontinuance of globulin therapy. Instead, the quality of function improved progressively from the second month onwards.

Effect on white blood counts. The average white cell count per day for the first 105 postoperative days was higher in each succeeding series (Fig. 7). This probably resulted from the increasingly cautious use of azathioprine described above.

The influence of ALG on the lymphocyte differential fractions and the total lymphocyte counts of patients being treated preoperatively solely with this agent was described previously (Iwasaki *et al.*, 1967). There was a tendency for lymphopenia to

develop after injections but this effect was highly variable. Studies of the peripheral lymphocytes in patients after transplantation were even more difficult to interpret because of the complex immunosuppressive regimen being employed. For example it has been shown (Starzl *et al.*, 1965) that there is a direct correlation between the quantity of steroids used after transplantation, and the degree of postoperative lymphopenia. As a consequence, comparisons in the present study between the retrospective control cases and those treated with ALG were invalid in that the latter patients received far less prednisone. With this reservation in mind, it is noted in passing that the percentage of lymphocytes in the peripheral smear was approximately the same in each of the control groups as well as in the ALG-treated patients (Fig. 7). The total peripheral lymphocyte counts per day were slightly higher in the ALG group, being 1,780/mm.^{3} as compared to 1,640, 1,510, and 1,410/mm.^{3} in the three control series.

Rejection. Seven of the 19 patients with satisfactory operations have never experienced rejection. Secondary deteriorations of renal function occurred in the other 12. However, a rise in BUN after the initial diuresis to as high as 150 mg./100 ml. occurred in only one case (Fig. 8), and to as high as 100 mg./100 ml. in only one other. In the remaining patients, the maximum secondary rise in BUN did not exceed 67 mg./100 ml. These rejections were all easily reversed, as can be appreciated from the composite postoperative function curves of the first eight patients (Fig. 6). Furthermore, delayed rejection has not been seen in any of the ALG-treated patients.

Clinical toxicity. All patients had pain at the site of the intramuscular injections. This was often severe and in a few cases narcotics were required. The complaints were usually greatest with the first few injections. Later, most of the patients were not prevented by the discomfort from carrying on their regular activities. Swelling and oedema were regularly observed, reaching a peak after three to six hours. It was at this time, rather than immediately after administration, that the pain was apparently most intense.

In addition, all patients developed fever at some time. Usually this was low-grade fever and often it would not be observed after every injection. In some cases, however, temperatures rose to more than 40°C even when steroid therapy was in effect. Four of

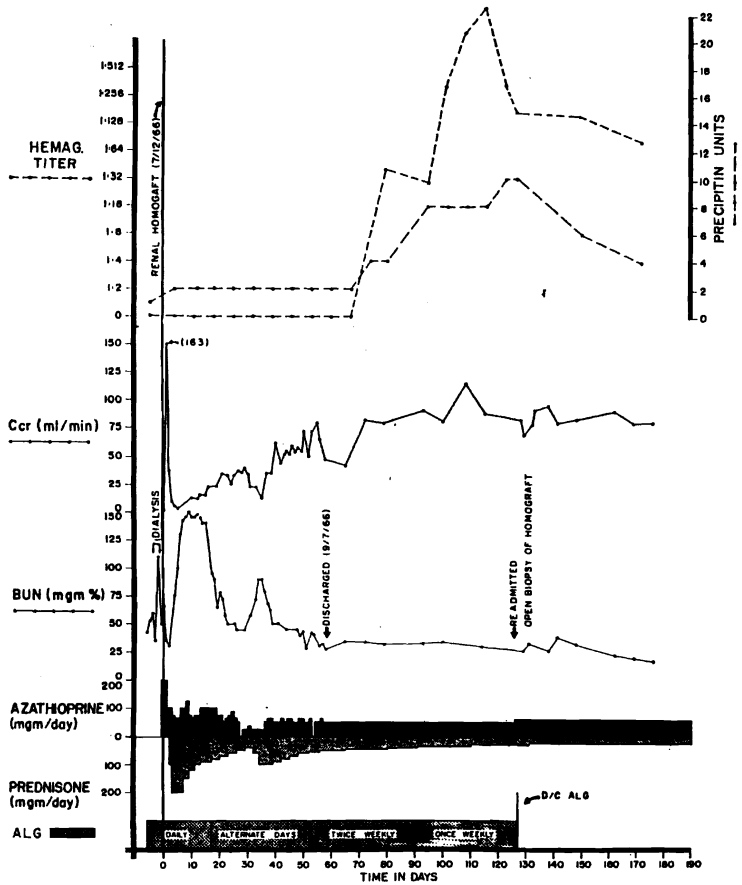

FIG. 8. The course of a patient who had the most severe rejection observed amongst cases treated with adjuvant ALG. Large doses of prednisone were initially required, but could later be withdrawn relatively rapidly as function improved. Note the late and modest increases in precipitin and haemagglutinin titres. The donor-recipient match according to Terasaki's analysis was a very poor one.

the 20 patients had other systemic reactions: three developed transient hypotension and air hunger; the fourth was briefly hypertensive. In three of these four patients, therapy was continued for a month or longer and then stopped either because of similar further reactions (two cases) or because the interval of planned treatment had ended (one case). In the fourth patient, the injections were immediately discontinued.

Throughout and after the course of globulin therapy, the urinary protein content was frequently determined. During the first few postoperative days or weeks, proteinuria was common, an early finding similar to that often noted in past cases. However, the abnormality regressed in all instances. The protein concentration in the urine of the first ten patients after 3 to $6\frac{1}{2}$ months is now 1 to 12 mg./100 ml.

Immunological and haematological evidence of toxicity. The lack of strict immunological specificity of ALG has been described elsewhere, including the fact that the antibodies agglutinate granulocytes to the same degree as lymphocytes (Iwasaki *et al.*, 1967). A theoretical possibility, therefore, might be that panleucopenia could be caused by ALG therapy. Such an eventuality would presumably require the specific condition of an additive interaction with other immunosuppressive agents, since the ALG preparation used has been shown to cause leucocytosis when given alone. In practice, the complication of leucopenia was seen only in the patient who died after the technically unsuccessful transplantation. In this case, it was reasonable to believe that azathioprine was primarily responsible for the bone marrow depression.

Observations of the peripheral platelet counts have, however, raised the possibility that thrombocytopenia may be caused by ALG. Eight of the 20 patients were noted at some time during their early postoperative course to have low platelet counts, from 50,000 to 150,000/mm.^{3}, at the same time as the peripheral leucocyte counts were normal or elevated. In only one case was the thrombocytopenia a significant problem. In this patient, the platelet counts were below 50,000/mm.^{3} for more than three weeks, during which time platelet infusions were required. After the ALG injections daily or every other day were reduced to twice a week, the platelet count slowly rose to normal. The batch of ALG then being used was examined by Drs. A. van Leeuwen and J. J. van Rood of Leiden and found to contain anti-human-thrombocyte activity.

The more specific information on ALG toxicity which was

sought with immunological measurements will be reported from the first eight patients for whom the longest follow-ups are available. Skin tests obtained before and after therapy showed an increased reaction in five of the eight cases after several weeks or months. The erythema, induration, or wheal formation were

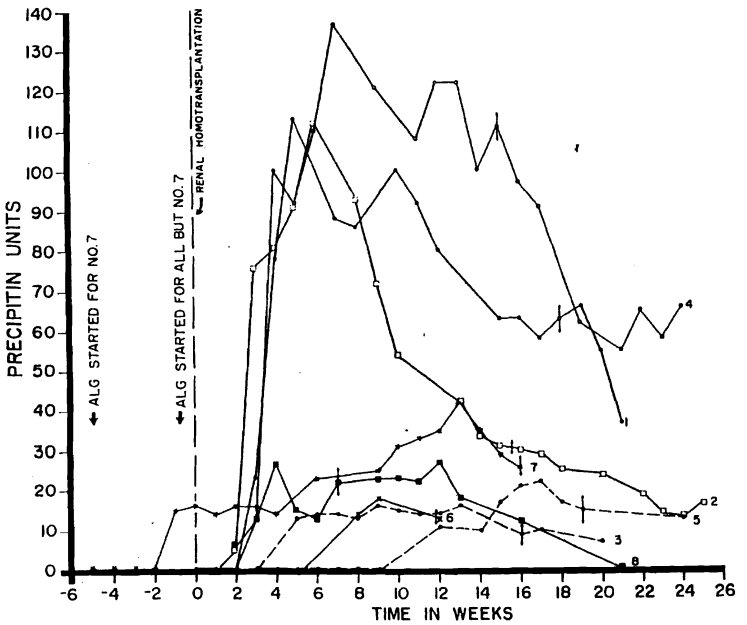

FIG. 9. Rises in precipitin titres in the first eight consecutive patients treated with antilymphocyte globulin. The vertical arrows indicate the last dose of ALG given in each case; note that the titres had often begun to decline before this time.

unchanged in the other three, with testing weekly or every other week.

All eight patients developed increases in precipitin titres, beginning from two to nine weeks after the beginning of ALG therapy (Fig. 9). The timing and magnitude of the rises appeared to be influenced by several factors. The greatest and earliest precipitin activity was in three of the four patients who did not receive steroids until 29 to 43 days after transplantation (Cases 1, 2,

and 4). In these patients, the precipitin titres fell after prednisone was begun in doses of 0·31 to 1·37 mg./kg. per day. Two of these three patients who had developed anaphylactic reactions subsequently received ALG for four to eight weeks without further difficulty; the third patient never had a reaction. The other five patients, in whom the precipitin increases were either later or of lesser magnitude, were treated in four instances with steroids beginning within the first two postoperative weeks. These findings suggested that prednisone therapy could reverse the precipitin response in situations where the foreign protein was continuously administered, or that alternatively the response could be attenuated if steroids were instituted at an earlier time.

However, a second feature of the precipitin response deserves special emphasis since it did not follow the institution of steroid therapy. Three of the four patients who received prednisone from the first few postoperative days onwards nevertheless developed a positive precipitin test (Cases 5, 6, and 7). The precipitin titres subsequently fell in spite of the fact that the steroid doses were then being reduced. The latter observation suggests that the altered late host reaction to the foreign protein described above, although influenced by steroid therapy, had a tendency to occur spontaneously.

The development of titres against sheep red blood cells tended to follow the same curve as the precipitin measurements, although somewhat more sluggishly. The highest titres seen were 1:256 (two cases) and 1:512 (one case). Two of these three patients were those who developed anaphylactic reactions with hypotension, and the third was a patient who had a transient bout of hypertension just after injection.

Altered host reactivity. Before ALG treatment was begun, Drs. Frank H. Brunstetter and Henry N. Claman studied eight of these patients with skin tests to *Candida albicans* (1:100), histoplasmin (1:100), mumps (1:100), trichophyton (1:100) and intermediate strength purified protein derivative (PPD) (10 T.U.). Tests were read 24 and 48 hours after intradermal injection of 0·1 ml. test material.

There were five positive reactions, one to PPD, three to mumps and one to trichophyton. All skin tests became negative within three or four days after ALG treatment began, thus demonstrating that the antilymphocyte globulin being used prevented the

TABLE I

FLUORESCENT AND ELECTRON MICROSCOPIC OBSERVATIONS ON THE GLOMERULI OF EIGHT RENAL HOMOGRAFTS IN PATIENTS TREATED WITH HORSE ANTIHUMAN-LYMPHOCYTE GLOBULIN

	Electron microscopy			*Fluorescent microscopy*					
	Thickening of glomerular capillary basement membranes			*Deposits demonstrated by fluorescent antibody technique*					
							Complement		
Patient	*Subendothelial*	*Subepithelial*	*Fusion of epithelial foot processes*	*IgM*	*IgG*	*Horse globulin*	β_{1A}	β_{1C}	*Fibrinogen*
LD 107	±	o	±	+	o	o	o	o	o
LD 108	o	o	±	o	o	o	o	o	o
LD 109	±	o	+	+	o	o	o	o	o
LD 110	+	o	+ +	+	o	o	+ +	+ +	+
LD 111	+ +	o	±	+	o	o	o	o	o
LD 112	o	o	±	o	o	o	o	o	o
LD 113	+	o	+ +	±	o	o	o	o	o
LD 114	+	o	±	+	o	o	o	o	o

o = negative; ± = slight in amount; + = moderate in amount; + + = marked in amount.

FIG. 10. Biopsy of a renal homograft four months after transplantation (patient LD 111). The basement membrane of a glomerular capillary loop is thickened by a subendothelial deposit of amorphous material (x). Fusion of the epithelial foot processes (f) is slight. The mesangial cells (mes) and matrix are increased in number and amount. cap = glomerular capillary lumen; end = endothelial cell lining capillary; cp = visceral epithelial cell; us = urinary space. × 8,200.

To face p. 22

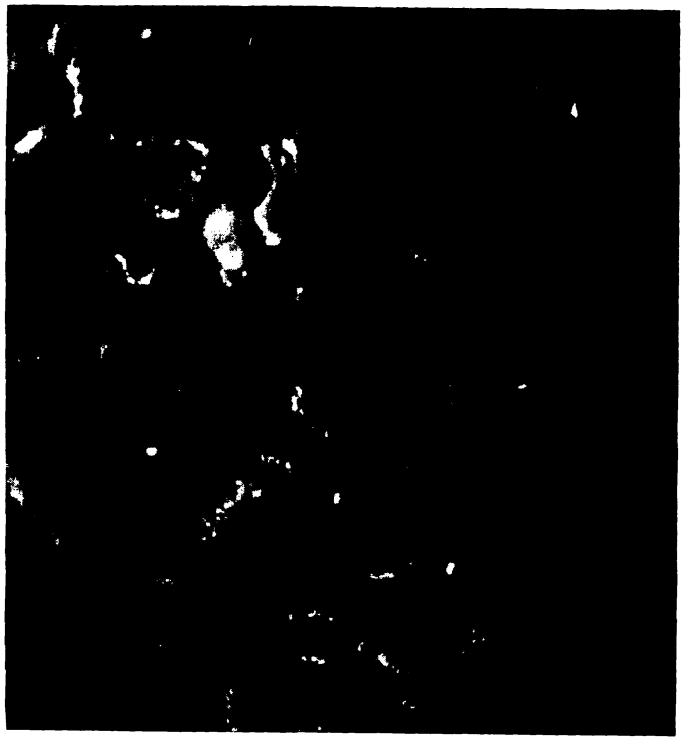

FIG. 11. Same renal homograft as was shown in Fig. 10. Section of biopsy treated with fluorescein-labelled goat antibody to human immunoglobulin M. There is a delicate granular and linear localization of IgM in the glomerular capillary walls. (By courtesy of Drs. Andres, Hsu and Seegal.) × 500.

expression of previously established delayed hypersensitivity. None of these patients had striking lymphopenia.

Nevertheless, the only serious infectious complication was pneumonitis in the patient who died after the technically unsatisfactory transplantation. There was also one example of herpes zoster, and several minor upper respiratory infections.

Other morbidity. Two of the patients who are still alive required reoperation for urological complications. In one, a uretero-pelvic stricture necessitated conversion at the ninth postoperative week of the ureteroneocystomy to a ureteropelvostomy. In the other, the tip of the homograft ureter sloughed within the ureteroneocystostomy tunnel and a uretero-ureterostomy was required after 13 days. Both patients recovered promptly.

Histopathological studies. Subendothelial accumulations of amorphous material were present in the glomerular capillary walls of six of the eight renal homografts biopsied 108 to 145 days after homotransplantation (Table I), but only in four of the kidneys could any thickening of the basement membranes be detected by light microscopy. The change was focal and in every glomerulus normal loops were present. The density and compactness of the deposits varied (Fig. 10); in one kidney it was as though the lamina densa had been split and a broad band of loosely arranged material inserted between the two layers. Mesangial cell processes extended into a few of the thickened basement membranes. There were no subepithelial deposits.

Immunofluorescence of the six affected kidneys demonstrated glomerular capillary basement membrane localization of IgM, generally in a linear pattern (Fig. 11); one homograft (LD 110) also had glomerular basement membrane deposits of β_{1A} and β_{1C} globulin and fibrinogen. No localization of IgG or horse globulin was detected.

In all the kidneys with glomerular capillary basement membrane changes there was some increase in the number of mesangial cells and the amount of mesangial matrix; the epithelial, endothelial and mesangial cells possessed increased numbers of free ribosomes, cytosomes and cytosegresomes and larger amounts of rough endoplasmic reticulum. In the four most severely damaged homografts some of the capillary lumina were narrowed by swollen endothelial cells. Neutrophil polymorphonuclear leucocytes were present in the glomerular capillary loops of three of

the latter transplants. In each of the eight kidneys there were areas in which the epithelial foot processes were fused, but this change was only pronounced in two of the grafts.

Although most of these renal homografts showed some vascular damage in the form of subendothelial collections of hyaline in arteriolar walls and fibrous intimal thickening of interlobular arteries, in four of the kidneys these changes were minor and affected only a few vessels. In only two (LD 107 and 111) were they at all marked.

Dense focal collections of mononuclear cells were present in seven of the homografts and were frequent in four of these. Up to 40 per cent of the cells had pyroninophilic cytoplasm. In two of the grafts the cytoplasm of some of the infiltrating cells stained positively for IgG, while others were positive for IgM; in three other grafts only cells that stained for IgM were found. Ultrastructurally some of the cells were plasma cells with elaborate rough endoplasmic reticulum and others were lymphoid with abundant free ribosomes in the cytoplasm but no rough endoplasmic reticulum.

Late cases

In all four of these cases there was a fall in BUN with the institution of ALG therapy and a subsequent reduction in prednisone dosage. This apparently was due principally to a diminution of the catabolic steroid effect rather than to a change in renal function, since the plasma creatinine levels or the creatinine clearances were not comparably improved. However, the function was now relatively stable in spite of the drastic withdrawal of standard immunosuppression and this state has continued in two cases for several weeks or months after completion of the ALG course. In two of the three patients in which an infectious complication was present this was brought under control.

Three of these four patients are still alive from two to $3\frac{1}{2}$ months after discontinuance of ALG. The fourth was a 13-year-old recipient of a cadaveric homograft whose renal function was extremely poor when globulin therapy was begun. Her course was not particularly different from that in the other cases, but she developed pulmonary oedema due to cardiac failure and viral pneumonitis, had a cardiac arrest, and died after a post-transplantation survival of ten months. The homograft weighed 95 g.

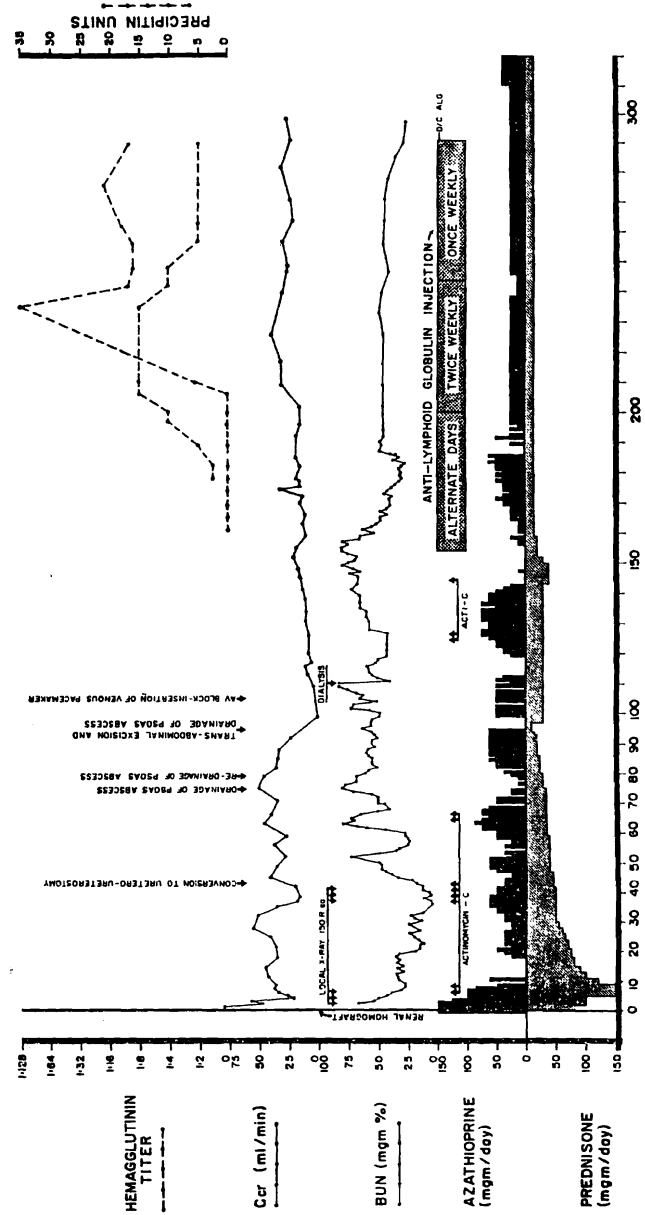

FIG. 12. Course of patient with a failing renal homograft who was treated late with antilymphoid globulin. During the first four postoperative months there were several potentially lethal postoperative complications which were made more grave by the need for heavy steroid therapy. Efforts to reduce the prednisone dosage resulted in deterioration of renal function. After institution of antilymphoid globulin therapy, renal function slightly improved despite a rapid decrease of the steroid doses, and this has been sustained since completion of the ALG course.

Microscopically many of the arteries were greatly narrowed by fibrous intimal thickening. This change was most severe in the interlobar and segmental arteries, several of which were completely occluded, and it was sometimes accompanied by rupture of the internal elastic lamina. The vascular lesions were associated with general interstitial fibrosis and widespread tubular atrophy. The surviving glomeruli showed thickening of the capillary basement membranes and increased amounts of mesangial matrix. It was thought that the arterial narrowing in this homograft had been caused by rejection.

The courses of two of the surviving patients are of interest. In one there had been multiple postoperative complications including pancreatitis, a left paravertebral gutter abscess, complete heart block, massive wound haemorrhage, and pneumonitis. To compound the problem, stable renal function could not be sustained with prednisone doses of less than 1 mg./kg. per day. During and after the course of ALG treatment the non-renal problems were controlled. In the meanwhile, the patient's renal function slowly improved (Fig. 12). Her condition is now excellent, $3\frac{1}{2}$ months after discontinuation of ALG therapy.

In another case, a slow but inexorable late rejection of a cadaveric homograft in a 13-year-old child could not be reversed with high-dose prednisone therapy. This effort was complicated by the development of acute pneumonitis. ALG therapy was started, and continued for five weeks. Steroid therapy was reduced and then stopped, and ultimately azathioprine and ALG were also discontinued when the pneumonitis became worse. During the ensuing eight weeks the patient had continuing although slowly deteriorating renal function. The pulmonary infection completely resolved, and retransplantation was carried out on 17 January, 1967. A second course of ALG therapy was started without untoward effects.

DISCUSSION

During the past seven years, considerable information has accumulated concerning the immunosuppressive and other properties of heterologous antilymphocyte serum (ALS). Waksman, Arbouys and Arnason (1961) were the first to show prolongation of homograft survival but the effect was an extremely weak

one, scarcely more impressive than in Woodruff's earlier negative experiments (1960). The subsequent investigations of Woodruff and Anderson (1963, 1964) catalysed widespread interest in the therapeutic possibilities of such antisera by demonstrating a striking protection of homografts in mice treated with ALS alone or in combination with thoracic duct drainage. Later, antisera of comparable or greater potency were developed by Gray, Monaco and Russell (1964), Monaco, Wood and Russell (1965), Levey and Medawar (1966*a*, *b*), Jeejeebhoy (1965), Nagaya and Sieker (1965), and Hoehn and Simmons (1966). In all these studies, the experimental model consisted of skin grafting in mice, rats, or guinea pigs which were treated, usually by intraperitoneal injection, with antisera raised in rabbits. The use of genetically controlled donor and recipient strains for transplantation permitted precise delineation of many of the features of the tested sera which have direct clinical applicability, as will be discussed later.

Nevertheless, certain intermediary steps were required before heterologous antilymphocyte products could be considered for clinical trial. It was necessary to demonstrate a beneficial effect of ALS after whole-organ transplantation in outbred animals, to determine the therapeutic schedules which were the most effective with the least toxicity, and to evolve practical techniques of administration which would be acceptable for use in man. This kind of information has been sought in dogs.

First, heterologous ALS raised in either the sheep (Mitchell *et al.*, 1966), rabbit (Abbott *et al.*, 1966; Lawson, Ellis and Hodges, 1966) or horse (Abaza *et al.*, 1966; Atai and Kelly, 1966; Huntley *et al.*, 1966; Pichlmayr, 1966; Starzl *et al.*, 1966, 1967), and given intravenously, intraperitoneally or subcutaneously as the sole therapy, has been shown to potentiate the survival of both canine renal and liver homografts. The results in individual experiments in all these studies were inconsistent. There were some extraordinarily long-term survivors but most animals eventually rejected their homografts. The same spectrum of response to therapy has been observed in the past with evaluation of azathioprine and other immunosuppressive agents, and is presumably the consequence of differences in the quality of chance histocompatibility matching in randomly paired mongrel dogs.

The effectiveness of subcutaneous injections in these studies was of the utmost importance since it was shown to carry far less risk

of acute toxicity from anaphylaxis than the other routes of administration. These observations also led to the decision that intramuscular therapy would be the clinical procedure of choice, since, unlike the situation in the dog, injections are poorly tolerated in the fatty subcutaneous tissue of humans. Such therapy could not be considered in man until a potent product was refined which could be given in small amounts. This was accomplished, as detailed elsewhere (Iwasaki *et al.*, 1967), by raising and selectively absorbing high-titre antiserum, identifying the immunoglobulin components which contained anti-white cell antibodies, and selectively removing these protein fractions. The resulting material was not only of a practical volume, but contained reduced total quantities of horse protein.

Concern about serum toxicity was not eliminated by the development of an improved antilymphocyte globulin. Although acute anaphylaxis has never been seen in our laboratory after subcutaneous injections of ALG in dogs, there were at least two other well-known and immunologically distinct kinds of reactions (Dixon, 1965) which could, by virtue of renal injury, defeat the purpose of this treatment.

The first of these was a Masugi-type nephritis in which a very rapid binding has been demonstrated of heterologous antikidney antibodies to glomerular antigen (Seegal *et al.*, 1962). With fluorescein-labelled antibody techniques the foreign protein could be seen for many months, mixed after the first week or so with secondary accumulations of host γ-globulin and β_{1C} complement. There was sound theoretical reason to fear this complication since ALG—at least that produced in our laboratories—is relatively non-specific. Its anti-white cell titre could be drastically lowered by absorption against kidney or liver cells as well as lymphocytes of the species against which immunization was conducted, indicating the presence of many shared antigens in all three kinds of tissue (Iwasaki *et al.*, 1967). Nevertheless, an acute nephritis, beginning abruptly after institution of ALG therapy, is not known to have been produced in any dogs in our laboratory.

An even greater unknown risk was that of serum sickness nephritis, which is not based upon a specific reaction of the heterologous antibody with host renal tissue. Instead, the injected foreign protein causes a host antibody response, with the result that soluble antigen-antibody complexes are formed peripherally,

coincident with a depression of β_{1C} complement. These complexes are mechanically trapped in the micro-circulation of the glomeruli where they provoke a secondary inflammatory reaction (Dixon, Feldman and Vazquez, 1961).

As with Masugi nephritis, the injury is not readily reversible since heterologous globulin, host γ-globulin, and β_{1C} complement can be identified in these kidneys long afterwards (Feldman, 1963; McCluskey, 1965). Such renal lesions were present in a significant number of normal dogs treated in our laboratories for longer than two weeks with relatively large doses of antilymphocyte plasma, serum, or globulin (Iwasaki *et al.*, 1967). In some of these animals in which canine precipitin antibodies against horse protein were studied, the titres had either increased little or not at all as compared to rapid and high rises of titre in control dogs treated with normal horse serum. These findings suggested that the well-documented self-antidotal properties of ALS or ALG which derive from the immunosuppressive action of these preparations (Levey and Medawar, 1966*a*; Gray *et al.*, 1966; Iwasaki *et al.*, 1967) were not necessarily sufficient to prevent serum sickness nephritis. It was hoped that the patients would escape this complication since they were given smaller quantities of horse protein and in progressively decreasing doses.

The above comments make it clear that the potential dangers of ALG therapy are by no means trivial, but they do not imply that hopes for the use of this agent in man should be abandoned. Instead, the vast amount of completed work in both the rodent and canine species has provided guide-lines within the limits of which a clinical trial seemed proper. A synthesis of this information into a therapeutic protocol for human use was recently presented, with a preliminary report of the first cases (Starzl *et al.*, 1967).

It was proposed, for several reasons, that ALG should be added as an adjuvant agent to therapy with azathioprine and prednisone: first, because of the imperfect immunosuppressive qualities of ALG when used alone; secondly, because the risks of serum reactions cited earlier made it prudent to limit its administration to the first few postoperative months; and finally because the incidence and severity of such reactions would presumably be favourably influenced by other immunosuppressive drugs.

This general position was strengthened by experimental evidence that ALG has an additive immunosuppressive effect when

used in conjunction with azathioprine (Starzl *et al.*, 1967) or other metabolites (Hoehn and Simmons, 1966), with prednisone (Levey and Medawar, 1966*a*), and, under special circumstances, with total body irradiation (Levey and Medawar, 1966*a*). The details of therapy were also influenced by the demonstrations in animals that homograft survival was improved if ALG was given for several days before as well as after transplantation (Huntley *et al.*, 1966; Monaco *et al.*, 1966; Starzl *et al.*, 1967), and that lymphopenia was not essential for an immunosuppressive effect (Abaza *et al.*, 1966; Huntley *et al.*, 1966; Mitchell *et al.*, 1966; Starzl *et al.*, 1966, 1967), for reasons best explained by the studies of Levey and Medawar (1966*a*).

The results with the clinical use of ALG under these carefully defined circumstances have been distinctly encouraging. There have been fewer deaths and less disability after renal homotransplantation than at any previous time in our experience, observations which become increasingly significant with each additional month of follow-up. It has been possible to maintain excellent homograft function with smaller quantities of the standard immunosuppressive drugs. The drastic reduction in the requisite doses of prednisone was of particular importance, since this agent has been thought to contribute more heavily than any other factor to both the delayed mortality and the morbidity after transplantation (Hill *et al.*, 1967). The freedom of the ALG-treated patients from infectious complications was striking.

The degree to which short-term treatment with heterologous immune globulin will influence the long-term prognosis after renal homotransplantation remains speculative, but there is reason to believe that any therapy which is of value during the early post-transplant period may confer a lasting benefit. This is not because of a continuing immunosuppressive effect. Rather, it appears to be due to an alteration which leads to a state of relative host-graft non-reactivity. This poorly understood change, termed "adaptation" by Woodruff and Woodruff, who were the first to show its development (1950), is the cornerstone upon which many of the advances in clinical homotransplantation are based, since it implies that the maximum need for immunosuppression is soon after operation. More recently, it has often been possible to maintain patients for years on drug doses no greater or even less than those which at an earlier time were quite

inadequate for control of rejection (Starzl, Marchioro and Waddell, 1963). Thus, although it could be argued that upward adjustments of azathioprine and especially prednisone dosage might be required at the termination of the four-month course of ALG, there was good reason to believe that this might not be the case. In point of fact, none of the eight patients who have been followed for two to four months since their last injection have required such adjustments.

Possibly it will eventually be found that immune globulin therapy is feasible on a much more chronic basis. For the moment, it is encouraging to note that ALG could be used for several months without prohibitive direct toxicity, the only consistent side effects being pain at the injection site and fever. Anaphylactic reactions which occurred in four patients were relatively minor and transient and in three of these cases a number of subsequent injections were given. It is reasonable to believe that the concomitant use of other immunosuppressive drugs contributed to the safety of the globulin administration, a possibility which was supported by the facts that elevated precipitin titres declined promptly in three patients after late institution of prednisone therapy, and that such titres rose later and more sluggishly in other patients in whom prednisone was started at an earlier time.

The most reassuring information, however, came from study of the homograft biopsies. None showed anything suggestive of chronic serum sickness nephritis either by fluorescence microscopy or by electron microscopy. The only glomerular and vascular lesions found were those that have been frequently encountered in renal homografts treated in a conventional manner and that are generally considered to be the result of the host's reaction to the foreign tissue (Porter *et al.*, 1966, 1967). The infiltration by lymphoid and plasma cells was also no more dense than that often seen in homografted kidneys at about this time after transplantation (Hamburger *et al.*, 1965).

So far, discussion has been confined to the newly operated cases in which ALG was given from before the time of transplantation. The effect of globulin therapy upon the course of the other four patients who had late-failing homografts is not easy to assess. Renal excretion was not improved in any instance, but stabilization of function was observed in two patients despite reductions in prednisone therapy of a magnitude which had previously been

impossible without precipitation of a rejection. In a third case, homograft function continued for two months in the absence of all therapy until retransplantation could be performed. It seems unlikely that ALG therapy can benefit many such patients unless it is given at an early time, before irreversible injury has occurred. Under these circumstances it may be possible to retain residual function with considerably less stringent prednisone therapy. Benefit of this kind would not be surprising since it has been shown that ALS can erase the immunological memory of presensitized animals (Levey and Medawar, 1966*a*; Monaco *et al.*, 1966), and because the abolition of pre-existing delayed hypersensitivity skin reactions in patients has been demonstrated within a few days of the institution of ALG therapy.

SUMMARY

An antihuman-lymphocyte globulin (ALG) was prepared from the serum of immunized horses for intramuscular administration in man. From June until December, 1966, it was used as an adjuvant immunosuppressive agent for the treatment of 20 patients before and after renal homotransplantation in accordance with guide-lines derived from previous animal experiments. The ALG was added to therapy with azathioprine and prednisone, and its use was restricted to the critical first four postoperative months. During the preoperative period when it was used alone, the ALG invariably abolished pre-existing delayed hypersensitivity skin tests of various kinds, an action which was not dependent upon lymphopenia.

Nineteen of the patients are alive and well; the only death resulted after a technical accident in a patient whose homograft also developed the same acute glomerulonephritis as had originally afflicted his own kidneys. Severe rejection was not observed. The doses of both azathioprine and prednisone used were less than in any previous series of transplantations at our institutions. There has been only one serious infectious complication in the entire series. Renal function in all ten of the patients who received transplantation more than three months ago is normal. In four more patients ALG therapy was used for patients with late-failing homografts; the results in these cases were equivocal.

The question of foreign protein toxicity was examined. Delayed but definite increases in precipitin and antisheep red cell titres were regularly seen. Relatively minor anaphylactic reactions occurred in four patients, but it was possible to continue therapy in three of these. Homograft biopsies after 108–145 days in the first eight newly transplanted cases were examined with electron microscopic and labelled-antibody techniques. There was no equine protein in any of these eight homografts, nor was there other evidence of "foreign protein nephritis".

It is concluded that the adjuvant use of heterologous ALG has improved the early course after human renal homotransplantation, and that this has been possible without excessive risk from the acute or delayed complications of serum sickness.

Addendum, 20 March 1967: The situation described in the text has not changed since the original manuscript was submitted. The patients then living are all still well.

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DISCUSSION

Gell: As the cytotoxicity of serum of this type depends on complement, the absence of the Fc fragment would presumably lead to its being both less immunogenic and less cytotoxic. Has anyone tried chopping that fragment off with papain?

Russell: Dr. Henry Winn in our laboratory has removed the Fc fragment of ALS by papain digestion, but so far the material is only in a bottle and not in an animal.

Gell: Is the pain patients feel after injection due to a local Arthus reaction?

Starzl: The pain is worst with the first injection; after a few days the patients generally tolerate it relatively well. Later, those patients who developed anaphylactic reactions reported that they had no pain at all.

Russell: We have treated one patient for almost eight weeks with three injections of 2·5 ml. of a purified IgG fraction of ALS each week. In her case the local reaction was indeed markedly less as treatment continued. One would hope that this lessening reaction might be a regular occurrence —that as the immunosuppressive effect of ALS goes into force the local as well as systemic immunological responses would be less.

Starzl: Dr. Nobura Kashiwagi, who has been working with us, has found that virtually all the precipitins which developed in the patients treated with ALG were directed against the α- and β-globulins. Since most of the desired anti-white cell antibodies are in the IgG fraction which evoked little precipitin response, it seems likely that the pure IgG preparations will be safer than the product now being used.

Woodruff: We have only treated one patient with horse antihuman globulin, and this was a girl in whom irreversible changes had already taken place. The graft had been in place for a year so we were not really expecting any miracles but we wanted to establish the safety of the procedure. This girl first had a course of intramuscular injections for about eight days and she had local pain and considerable red swellings at the site of the injections, despite the fact that she was on fairly heavy doses of steroids at the time. Two months later we gave her a second five-day course intravenously because of these complications, but to this she showed no reaction and had no pain whatever. I don't think this treatment achieved any dramatic changes in the transplant and I am not particularly advocating the intravenous route, but it was interesting that it was so well tolerated. We were struck by the fact (Abaza *et al.*, 1966, *loc. cit.*) that a serum-sickness-like phenomenon occurred in the dog after crude horse serum (whether ALS or normal serum) was administered, but whereas this recurred in a much more severe form at eight days or so in control animals in which normal horse serum was used, there was no later reaction in animals which

received ALS; ALS thus seemed to protect against one at least of its own potential complications.

James: Ultracentrifuged IgG preparations might prove less toxic and less immunogenic. It is fairly well established that the isolation and sterilization of IgG can produce undesirable configurational changes. In addition might it not be advantageous to give an initial large injection of antilymphocytic globulin? This might produce partial tolerance in addition to incapacitating a large proportion of the lymphocytes, thus reducing the chance of reaction to subsequent injections.

Humphrey: From the comments made so far, would it be fair to say that the precipitating antibodies that have been found have been against α- and β-globulins and not against immunoglobulins of the injected material?

Starzl: I didn't mean to go that far. There are some anti-IgG precipitins but they are in the minority.

James: Our preparations contain only IgG and IgG(T) and immune elimination occurred, suggesting that antibodies are being produced against these proteins. However the use of a stronger antiserum might completely suppress humoral antibody formation, thus preventing immune elimination.

Balner: Serum from monkeys treated with purified IgG did not contain demonstrable precipitins, whereas serum from monkeys treated with whole serum usually contained precipitins against rabbit proteins but not against γ-globulin.

Monaco: Some of our recent evidence suggests that the response to ALS depends on the size of the initial dose. A very large dose with a good immunosuppressive effect on the allograft reaction produces no response to the serum, but if we give very small doses of ALS and test much later after serum administration, we can show antibody to the serum. In the small number of normal human subjects to whom we have given ALS, we have been able to show formation of antibody specifically to the IgG. Also, we have now two dogs left in our series which have received ALS for one year and eight months and we have not been able to demonstrate any particular abnormality on electron microscopy.

Lance: We have some recent evidence that the IgG component of ALS may be handled somewhat differently from the IgG component of normal rabbit serum. When we did antigen elimination curves, the IgG from normal rabbit serum was eliminated by non-immune decay, whereas that from ALS instigated an immune type of elimination.

Medawar: A most important practical point is obviously the purity of the ALS. It seems quite practical to purify the ALS in the sense of removing from the IgG fraction irrelevant constituents like the other immunoglobulins, albumin and so forth. But I don't see how, on a large scale, one is going to remove the active antibody, or group of antibodies, in the IgG fraction from other and perhaps undesirable antibodies. The way to do

that is not to have irrelevant antigens present in the first place, and therefore to raise antisera not by injecting whole cells but by injecting fractions of cells. There seems no reason why one should go to all the trouble of inducing the formation of high titres of antibodies which one doesn't want. We know that certain lymphocyte cell fractions will produce ALS-type antibodies extremely well, and indeed better than whole cells, and I should have thought this was the kind of thing that ought to be done now in clinical practice. Those who are injecting ALS into human beings should tell us what material they are using to raise their antibodies.

Starzl: We immunized with whole cells. At first, we used lymphocytes from thymuses and lymph nodes. It was unusual to retrieve as many as a billion cells from a cadaveric donor. We eventually began to use spleens in order to get larger doses of antigen. A cadaveric human spleen ordinarily provides 40 to 200×10^9 cells. Before making this change, we could not obtain leucoagglutinating titres much higher than 1:64 in our horses. Afterwards, the titres rose within a few weeks to as high as 1:16,000 or even greater.

Monaco: We used lymphocytes from lymph nodes as the immunizing material. When we raised our antihuman lymphocyte serum in horses we found that all horses had very large amounts of circulating IgG, so that we were adding our specific antibody to a large pool of IgG and recovering this in the purified preparations. Our material from the horse had 45 mg. protein/ml. If we used young rabbits with very low levels of IgG to start with and then immunized them, we could get a material with a comparable lymphoagglutinin with 10 mg. protein/ml., so that we subsequently administered a much smaller amount of protein. We still get irrelevant antibodies, but we have been able to reduce the amount of protein.

James: The question of the species in which the antiserum is raised is a very important one. First of all we have to consider the ability of the immunized species to produce antibody against the injected lymphocytes. Secondly there is the question of the immunogenicity of the antilymphocytic antibody preparations in the animal receiving this form of treatment. In our experiments the rabbit antirat material was more effective than the horse antirat lymphocyte IgG preparations. Furthermore the rabbit IgG is less immunogenic in the rat than the horse preparations and so is less readily eliminated by immune mechanisms. The rabbit antibody may therefore remain in the body for a greater length of time and thus exert a prolonged effect.

Russell: A priori, this makes sense, but I don't think we know yet whether immunogenicity of the ALS preparation is a good or a bad thing. It may turn out that immunogenicity of the heterologous protein is a very important aspect of its mechanism of action. This question of

species selection for immunization is, therefore, rather subtle and interesting.

Woodruff: We used thoracic duct lymphocytes to raise all except our human sera, not because we thought this was necessarily the best way to get the sera, but because we thought it was interesting to see what we got by using a relatively homogeneous cell population. To go a bit further and use cell membranes seems to me of great immunological interest, and it may well also be of considerable advantage in a production line procedure. But I want to ask Sir Peter Medawar whether he thinks that by using this, one is going to refrain from making, as he seemed to suggest, some really significant unwanted antibodies, and if so, to what they would be antibodies?

Medawar: They would be antibodies to every ingredient of, shall we say, a human lymphocyte that was not also possessed by a horse.

Woodruff: Are there other effective antigenic determinants in the human lymphocytes that are going to immunize the horse, apart from the ones presented in your membrane preparations? That is really my question.

Medawar: And of course I don't know the answer. It is simply a matter of principle: if one is having some trouble with the sera, and if impurities in the sera are part of the trouble, then one should take all possible steps to remove the impurities, including the avoidance of raising antibodies against antigens that are not implicated or not known to be implicated.

Woodruff: An important general point is really at issue here. The human work has to tag along behind basic biological observations and this sort of question I have put is the kind of thing that would be answered in animal systems. Clinicians should not try to be too pure until biologists have shown them the way.

Medawar: There is very little danger of that! Animal experiments have already shown, for example, that crude so-called membrane fractions from lymphocytes are extremely good ALS producers (Levey, R. H., and Medawar, P. B. [1966]. *Proc. natn. Acad. Sci. U.S.A.*, **56**, 1130).

Batchelor: Professor Starzl, what happened to the haemagglutinin titres and your horse sera when you switched from using thymus and lymph node cells as your antigen to using spleen cells? It is quite a severe practical problem getting rid of the haemagglutinins, which I would have thought was a desirable thing to do, and I would have expected very high haemagglutinin titres to appear when you switched to using spleen.

Starzl: The haemagglutinin titres were sometimes as high as 1:100,000. These can be absorbed with human red cells without reducing the anti-white cell titre. It is a practical problem only for the technician who has to do it. It takes about three days.

Taub: The haemagglutinin titres will not specifically indicate how much anaemia one might see, especially in a patient who is also receiving steroids. The haemagglutinin titre, even of hetero-antibodies, is not necessarily related to the degree of haemolysis.

Starzl: Not necessarily, but in fact it was a terrible problem when we were using the antidog serum. When we began a dog on a course of unabsorbed ALG or ALS, the haematocrit would often fall as much as 25 or 30 per cent in a few days.

Taub: With a highly haemolytic serum, correlation of degree of anaemia with agglutinin titre becomes even less meaningful. Also, the dogs were not getting steroids. With your absorbed sera, what was the transfusion requirement in these patients?

Starzl: In patients we have never seen anaemia resulting from ALG. We did have a bad batch of ALG which hadn't been completely absorbed. We used it for a few days until we found that it had a haemagglutinin titre of about 1:1,000 as well as a haemolysin titre of 1:256. We stopped using it. There were no apparent harmful effects.

Woodruff: But there is no doubt that it makes both rats and dogs extremely anaemic. It would be a mistake to gloss over this.

Taub: We can make our mice anaemic even with the red-cell absorbed serum.

Calne: I am sure that Professor Woodruff was not suggesting that antilymphocytic serum should not be used clinically. We have used Imuran (Burroughs Wellcome) and steroids for several years now, and we have no idea how they act, but we know that they do act. Professor Starzl's early experience with antilymphocyte globulin demonstrates that it is a useful adjunct to immunosuppression. The application of ALS to clinical transplantation should therefore go ahead in carefully studied series as quickly as possible, since patients are perhaps now being denied therapy that might benefit them even at this stage of our knowledge.

Watt: Sir Peter has mentioned that lymphocyte capsular material can be used as the antigen instead of whole lymphocytes. Since the latter include the cytoplasmic and nuclear proteins, some of which must be common to the cells of other tissues, I wonder if antibodies to these proteins might be responsible for the appearance of precipitation which Professor Porter found in the glomerular epithelium cells from Professor Starzl's patients. Professor Porter, have you examined other tissues for evidence of this precipitation?

Porter: Yes, as far as possible. In the eight human renal allografts that were biopsied we found deposits on the endothelial side of the glomerular capillary basement membranes. But similar accumulations occur in transplants that have not been treated with antilymphocyte globulin, and in some of these patients deposits have been found in the walls of the renal arteries and arterioles as well as in the glomeruli, but never in other tissues (Porter *et al.*, 1966, *loc. cit.*). In the experiments with dogs that preceded

the clinical use at Denver of ALG, chronic serum sickness nephritis occurred in 13 of 21 animals given ALS or ALG for 17 days or longer. Dense subepithelial collections of equine globulin, canine IgM and β_{1C} globulin were produced on the glomerular capillary basement membranes of the animals' own kidneys. Although we examined many other tissues from these dogs, deposits were not found elsewhere (Iwasaki *et al.*, 1967, *loc. cit.*).