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OCCURRENCE AND CROSS-REACTIVITY OF HETEROPHILE ANTIBODIES AND ANTI-KIDNEY ANTIBODIES IN KIDNEY TRANSPLANTED PATIENTS AND PATIENTS WITH RENAL DISEASE

S.-E. SVEHAG, R. OLANDER AND K.-G. SUNDQVIST

Department of Immunology, National Bacteriological Laboratory, Stockholm and Department of Medicine V, Sahlgrenska Hospital, Gothenberg, Sweden

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SUMMARY

Increased titres of heterophile antibodies to rat erythrocytes occurred in twelve of twenty-seven patients after renal transplantation. In seven of these twelve patients the titre rise appeared to be associated with rejection. Heterophile antibody formation showed no consistent kinetic pattern after transplantation and no definite relationship between rise in antibody titre and rejection can be claimed. Patients with very high heterophile antibody titres were however prone to rejection.

Heterophile antibodies to rat erythrocytes cross-reacted with human and monkey kidney cells and a subpopulation of these antibodies also with human B erythrocytes. The antibodies were not of the Forssman or Paul-Bunnel-type and their appearance could not be related to ABO or HL-A incompatibility. The heterophile antibodies, primarily of IgM class, are suggested to be produced in response to B-substance related antigens in Gram-negative bacteria and non-HL-A isoantigens.

Approximately 35% of transplantation sera or sera from patients with kidney disease had IgG antibodies reacting with human and monkey kidney cells, human thyroid cells and A and B erythrocytes. Anti-kidney IgG antibodies in certain sera cross-reacted with rat erythrocytes. One-third of the patients with renal disorders had increased heterophile antibody titres.

INTRODUCTION

In early studies of transplantation immunity (Brent, 1958) it was generally believed that serum antibodies played no essential role in allograft rejection. Subsequently, it has been possible to demonstrate the appearance of circulating antibodies as a consequence of allografting (Abeyounis *et al.*, 1964; Milgrom *et al.*, 1966; Iwasaki *et al.*, 1967; Almgård & Svehag, 1968) and it is now widely accepted that antibodies to isoantigens in the transplant participate in certain types of hyperacute (Kissmeyer-Nielsen *et al.*, 1966; Williams *et al.*, 1968; Patel & Terasaki, 1969; Bergentz *et al.*, 1970) and acute kidney rejections (Clarke *et al.*, 1968). Rising titres of heterophile antibodies against rat erythrocytes after skin or

kidney transplantation was first reported by Rapaport *et al.*, (1967, 1968) and Almgård & Svehag (1968). The genesis and cross-reactivity of these heterophile antibodies is however still to large extent undefined and their role in allograft rejection has recently been debated (Tiong & Morris, 1972a; McDonald *et al.*, 1971).

As our earlier studies in dogs indicated an association between rises in heterophile antibodies and transplant rejection the assessment of such antibodies has been extended to human kidney transplant recipients. The occurrence and cross-reactivity of anti-kidney cell membrane antibodies in sera of transplanted patients or patients with renal disorders was also studied by the sensitive mixed haemadsorption technique.

MATERIALS AND METHODS

Sera

Serum samples were obtained from forty-three (cadaver) kidney-transplanted patients from 1 week up to 2 years after transplantation, from thirty-three not transplanted patients with renal diseases, from twenty-four patients suffering from a variety of diseases not involving the kidneys and from seventy-two apparently healthy blood donors. All transplanted patients and the majority of the patients with renal disorders had received multiple blood transfusions. The sera were stored at −20°C.

Treatment with sulfhydryl compound

Selected serum samples from kidney-transplanted patients were diluted 1:2 in phosphate buffered (0·01 M) physiological saline (PBS) pH 7·2 and reduced with 0·2 M 2-mercaptoethanol at pH 7·5 for 2 hr at 20°C. A 10% molar excess of iodoacetate at pH 8·0 was then added and allowed to react at 30 min at 20°C. Excess reagents were removed by overnight dialysis at 4°C against PBS.

Absorptions

Aliquots of antiserum, diluted 1:5 to 1:10, and of the appropriate cells ($= 1 \times 10^7$) were incubated for 2 hr at 37°C and the cells removed by centrifugation. The absorption procedure was repeated once.

Erythrocytes

Erythrocytes from W/Fu rats, guinea-pigs, sheep, oxen and humans of blood group A, B and O were washed three times and stored at 4°C in Claus-Jensen buffer prior to use.

Cultured cells

Monkey (*Macaca fascicularis*), human or rabbit kidneys were dispersed with 0·25% trypsin and 5×10^6 cells were seeded in Roux bottles containing Hanks's solution with 10% bovine serum albumin and 0·5% lactalbumin hydrolysate. A confluent monolayer was usually obtained within a week.

Surgically resected human thyroids were dispersed and the cells cultured as described for the kidney cells. The cell cultures were confluent within 4 days.

HeLa, Hep-2, KB and Lu-M cultures were prepared from cell lines maintained at our laboratory.

Haemagglutination

In the early phase of the study haemagglutination tests were performed in tubes with heat-inactivated test sera diluted serially (1:1·5) in Scheibel's buffer containing 0·3% normal rabbit serum. A 0·5% erythrocyte suspension was used and the tubes were examined after 2 hr incubation at room temperature. In the latter part of the investigation heat-inactivated test sera, diluted serially (1:2) in Scheibel's buffer containing 0·2% bovine serum albumin, were examined using the Takatsy microtitrater.

Mixed haemadsorption

Mixed haemadsorption tests were performed with *Macaca fascicularis* kidney, human thyroid, rabbit kidney, HeLa, Hep-2, Lu-M and KB cells as target cells. Sheep anti-human globulin serum, human amboceptor and coated indicator cells were prepared as earlier described (Fagraeus *et al.*, 1965).

Immunosuppressive treatment of kidney recipients

The treatment was started in advance of transplantation with extracorporeal irradiation of the blood (ECIB) in some of the patients as described by Persson *et al.*, 1969. Azathioprine was given from the day of transplantation in an initial dose of 150–200 mg/day. Later the dose was adjusted according to the leucocyte count of the blood to avoid leucopenia below 3000 cells/mm^3. Prednisone-therapy was also initiated on the day of transplantation with initial doses up to 200 mg/day, which dose was reduced day-by-day to approximately 1 mg/kg body weight 14 days post-operatively. The maintenance dose of prednisone was kept at 7·5–20 mg/day. Rejections were treated with unchanged or lowered doses of azathioprine to avoid bone marrow depression. Prednisone medication was increased to 75–125 mg/day. In some cases 200 μg/day of actinomycin C was given up to a total dose of 1000 μg. Local irradiation (150 rads) of the transplanted kidney was given every second day.

RESULTS

Heterophile haemagglutinins in human transplant recipients, nephritis patients and normal blood donors

Sera from forty-three human recipients of kidney transplants were screened for heterophile antibodies to rat erythrocytes. The sera were divided into two groups. The first group of twenty-six serum samples was collected 1–5 weeks after transplantation (C) and the second group of seventeen samples ½–2 yr after transplantation (D). In this part of the study the haemagglutinin (HA) determinations were made in test tubes. The distribution of HA titres in these sera are shown in Fig. 1, left part. It can be seen that 20–30% of the sera gave HA titres exceeding 750, the higher percentage value obtained with sera collected more than ½ yr after transplantation when, in general, the immunosuppressive treatment of the patients was less intensive. In contrast, no sera in the two control groups (A + B), including twenty-two normal blood donors and twelve patients with a variety of diseases not involving the kidneys, had HA titres exceeding 750.

Another group of fifty-eight sera were examined using the Takatsy microtitrater (Fig. 1, right part). This group included sera from twenty-one patients with renal diseases which had received no or only limited immunosuppressive treatment (H), twelve sera from patients

with various renal disorders on more intensive immunosuppressive treatment (G), twelve sera from patients suffering from diseases not affecting the kidneys (F) and thirteen from normal blood donors (E). Only 6% of the samples in the two control groups and 8% of patients on intensive steroid and/or azathioprine treatment had HA titres exceeding 128 while 57% of sera from patients with renal diseases which had received no or only sporadic immunosuppressive treatment had titres above this level. In three of these sera titres as high as 2048–4096 were recorded.

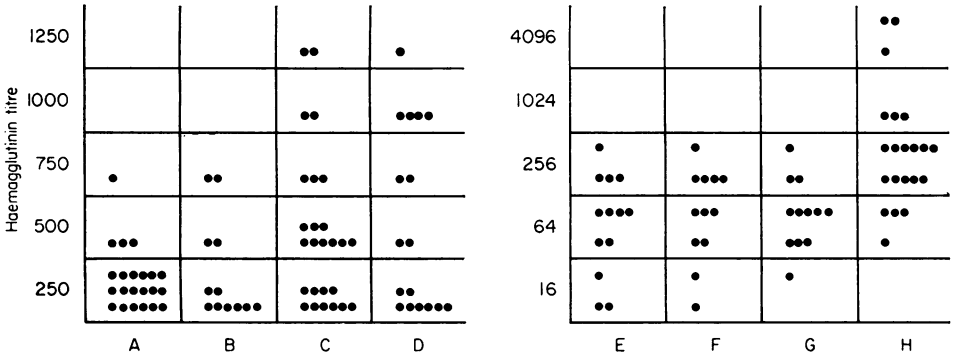

FIG. 1. Distribution of heterophile antibody titres against W/Fu rat erythrocytes in eight groups of in all 135 human sera. A, sera from normal blood donors; B, sera from patients with a variety of diseases not involving the kidneys; C, sera collected 1–5 weeks after kidney transplantation; D, sera collected ½–2 yr after kidney transplantation; E, sera from normal blood donors; F, sera from patients with a variety of diseases not involving the kidneys; G, sera from nephritis patients on strong immunosuppressive treatment and H, sera from nephritis patients on no or weak immunosuppressive treatment. Groups A–D were examined by the test tube technique, groups E–H using the Takatsy microtitrater.

Changes in heterophile antibody titres following transplantation and relationship to rejection

In ten cases up to a dozen serum samples were obtained within the first 2 months after transplantation and in a few cases also with varying time intervals before transplantation. The heterophile antibody titres did not follow any consistent recurrent kinetic pattern after transplantation. The titre could drop slightly (two patients), remain rather constant (three patients) or increase after 1–3 weeks (five patients) in spite of continued immunosuppressive treatment. The titre changes observed in sera from four patients in the last group are illustrated in Figs 2–4. In three of these patients elevated heterophile antibody titres appeared to be related to rejection crises.

Patient L.A. (Fig. 3) had HA titres around 100 prior to receiving the kidney transplant. Three weeks after transplantation the titres increased to reach a peak about a month after the operation. This occurred in spite of continuous azathioprine treatment. The serum creatinine increased as the antibody titres approached the peak titre. Repeated local X-ray treatment (600 rad) was therefore started 33 days after transplantation with a subsequent decline in both serum creatinine and agglutinin titres. Renal angiography performed twice during the period of increased serum creatinine levels showed no obvious signs of rejection.

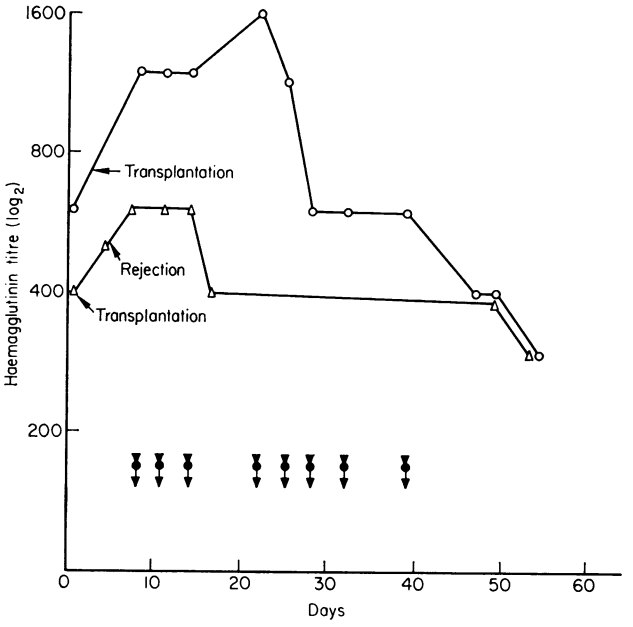

FIG. 2. Changes in titres of heterophile antibodies against W/Fu rat erythrocytes after transplantation in sera from two recipients. ▲, ●, titres after reduction—alkylation of sera.

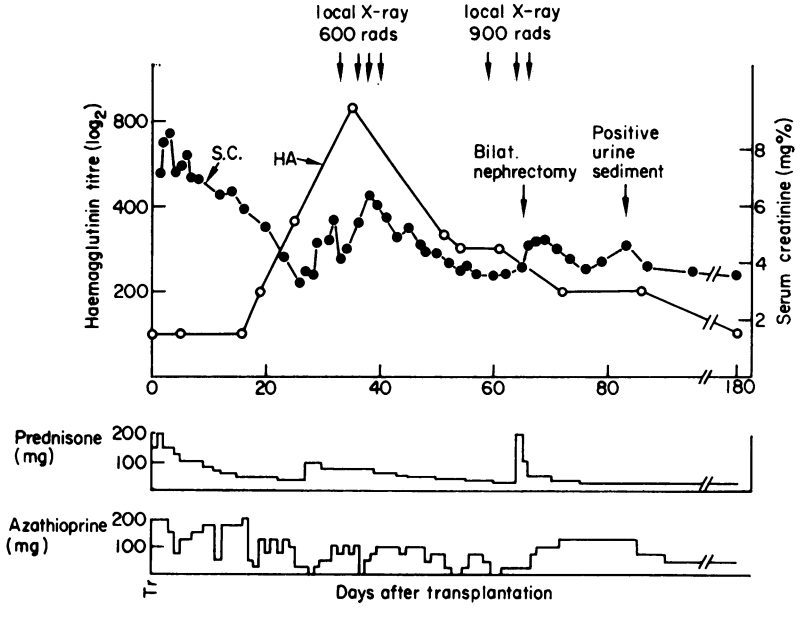

FIG. 3. Formation of heterophile antibodies to rat erythrocytes, serum creatinine levels and immunosuppressive treatment in a kidney transplanted patient. ●, Serum creatinine, ○, Heterophile antibody. (See also Results.)

A positive urinary sediment, rich in mononucleated cells, was however detected 2½ months after transplantation. Local X-ray treatment was administered again about 2 months following transplantation. After this treatment the serum creatinine levels have been rather constant (3·6–4·0 mg%) and the HA titres have remained at low levels (100–200) over a period of 3 months. The patient's own kidneys were not removed until 2 months after transplantation.

Patient L.J. (Fig. 4) received a D-match kidney transplant from her mother. Three weeks later the kidney was removed due to a strong rejection reaction. No agglutinin determinations were made during this period. The patient was kept on dialysis for 6–7 months. After 3 weeks of immunosuppressive treatment with azathioprine and ECIB

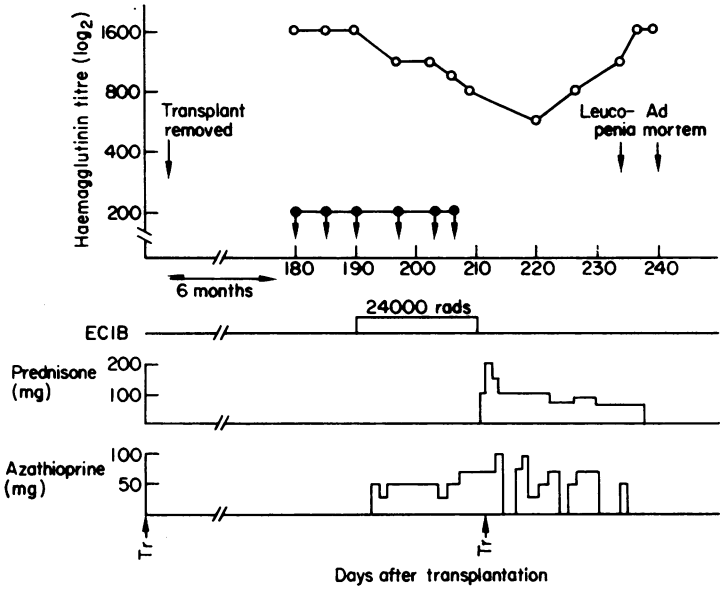

FIG. 4. Formation of heterophile antibodies to rat erythrocytes and immunosuppressive treatment in a kidney transplanted patient. Heterophile antibodies before (○) and after (●) reduction-alkylation.

L.J. was retransplanted with a cadaver kidney. The serum HA titres of this patient, which were high prior to the immunosuppressive treatment, declined slowly during the treatment. The haemagglutinins were primarily of IgM class. Three weeks after retransplantation the HA titres increased again. BUN and serum creatinine determinations indicated that the cadaver kidney functioned poorly. The patient developed leucopenia and died in Gram-negative sepsis three weeks after retransplantation.

Increased heterophile antibody titres were observed in seven out of seventeen transplant recipients more than ½ yr after transplantation. Three, or possibly four, of the patients with elevated titres showed chronic rejection reactions.

At this point it is also pertinent to comment the results in Fig. 6. Randomly collected sera from forty-seven nephritis (N) and kidney transplanted (T) patients were tested, simultaneously for heterophile antibodies to rat erythrocytes and anti-kidney antibodies

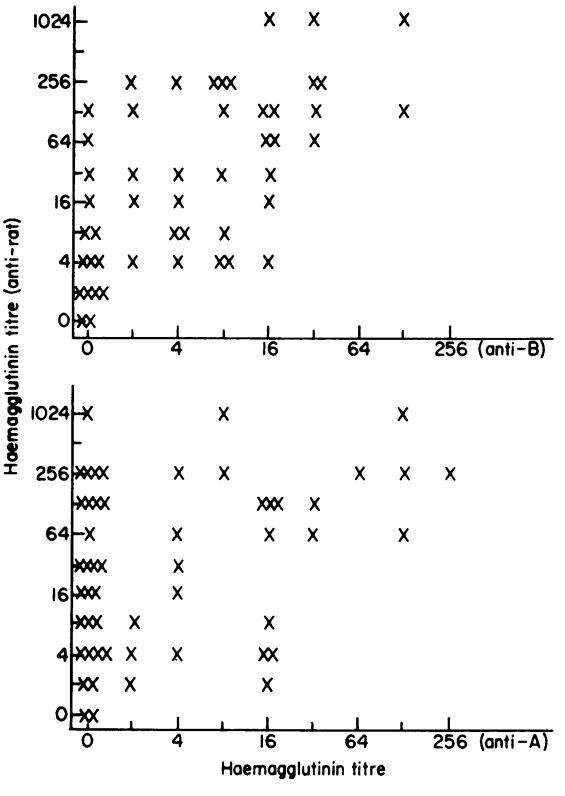

FIG. 5. Relationship between heterophile antibody titres against W/Fu rat erythrocytes and anti-A and anti-B activities in sera of fifty normal blood donors.

by the mixed haemadsorption technique. Subsequent examination of the heterophile antibody titres revealed that the twelve transplant recipients could be divided into two groups. The one group of seven patients with titres $\leq$200 showed no signs of rejection while the five recipients with heterophile anti-antibody titres $\geq$600 all were treated for acute (AR.) or chronic (CR.) rejection in close association with taking the serum sample.

Cross-reactivity and Ig class of heterophile haemagglutinins

Sera with agglutinins to rat erythrocytes had weak activity to sheep and ox erythrocytes. A few sera with high anti-rat titre (800–1200) agglutinated guinea-pig erythrocytes at dilutions of 200–400. The titres of most transplant recipient sera were distinctly reduced following absorption with cultured Macaca kidney or allogeneic human kidney cells and limited absorptions with rabbit kidney cells suggested a significant but weak titre drop (Table 1). In contrast, absorptions with HeLa cells (derived from a person with blood group O), human lung cells or O erythrocytes resulted in no clear titre decrease. Absorptions with human A or B erythrocytes gave variable results, in many cases causing a marked titre decrease, particularly when B cells were used (Table 1). No difference was observed between the absorbing capacity of Rh+ and Rh− cells. Absorption with rat erythrocytes abolished all the heterophile antibody activity to rat cells.

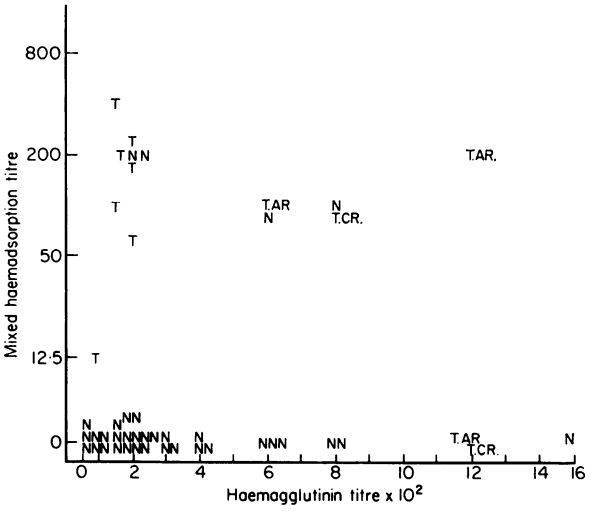

FIG. 6. Comparison between titration end points on monkey kidney cell monolayers and with W/Fu rat erythrocytes in sera from forty-seven kidney transplanted patients (T) or patients with kidney disease (N). AR, acute rejection; CR, chronic rejection.

TABLE 1. Effect of absorption with various cell types on the titres of hterophile antibodies to W/Fu erythrocytes in transplantation serae

Serum	Not absorbed	Human kidney	Human lung	A erythro-cytes	B erythro-cytes	O erythro-cytes	HeLa	Rabbit kidney
Gt 38	800	200	800	800	400	800	800	400
Gt 36	1200	200	1000	1200	100	800	1200	400
Gt 33	400	50	400	400	50	400	300	100
Gt 34	300	—	300	150	10	300	300	150

—, No absorption performed.

To further investigate the cross reactivity between human A and B substance and rat erythrocyte antigens sera from fifty normal blood donors of both sexes were tested against A and B erythrocytes and the correlation between these activities and the anti-rat erythrocyte titres of the sera was determined. The results (Fig. 5) suggested a weak correlation between anti-rat cell and anti-B activity. Furthermore, the rat erythrocytes reacted in immunofluorescence with human anti-B serum.

There was no correlation between the number of blood transfusions received by the kidney recipients and their heterophile HA levels. All recipients had received kidneys from ABO compatible cadaver donors. Although this was not systematically analysed there was no apparent correlation between the titre of heterophile antibodies and the degree of incompatibility for HL-A antigens.

Reduction and alkylation studies as well as centrifugation in sucrose gradients of sera from transplant recipients (Figs 2 and 4) indicated that the heterophile haemagglutinins were primarily of the IgM class.

Antibodies in human transplant recipients and nephritis patients demonstrable by mixed haemadsorption technique

Detection of antibodies to cell surface antigens in sera of transplant recipients and sera from patients with renal disease was also attempted by the mixed haemadsorption technique. Monkey kidney (*Macaca fascicularis*), human thyroid, HeLa, Hep-2, Lu-M and KB cells were used as target cells. Sporadic weak positive results, obtained with Hep-2 and KB-cells, showed no correlation to the heterophile HA titres in the same sera. No antibodies to HeLa or Lu-M cells were detected.

In contrast, 36% of sera from patients with renal disease and 35% of sera from kidney recipients reacted with Macaca kidney cells (Table 2). Practically all of these sera gave positive reactions also on human thyroid cells and there was a suggestive correlation between the antibody titres in the two cell systems. It is to be noted that approximately one-third of the sera in the liver disease control group also reacted with the kidney cells. The reactivity in the two remaining control groups (thyroid disease and normal blood donors) was 8–9%.

TABLE 2. Incidence of anti-kidney antibodies in sera from different groups of patients

Patient category	Number tested	Positive sera in mixed haemadsorption (%)
Kidney transplantation	49	35
Kidney diseases	27	36
Liver diseases	59	32
Thyroid diseases	22	9
Blood donors	33	8

Sera were tested in dilution 1/10.

The antibodies recorded in the mixed haemadsorption test were primarily of the IgG class. When the same sera were tested by immunofluorescence on suspended kidney cells using mono-specific anti-IgG and anti-IgM conjugates, the predominating activity was also due to IgG antibodies.

Cross-reactivity of antibodies recorded by mixed haemadsorption

No correlation was observed between mixed haemadsorption anti-kidney cell IgG antibody titres and titres of agglutinins to rat erythrocytes either in sera from transplanted patients or from patients with renal disease (Fig. 6). To investigate this further, absorptions were carried out with rat erythrocytes. Since sera which were strongly positive in mixed haemadsorption on kidney cells also agglutinated human A and B erythrocytes at rather high titres absorptions were also performed with A and B cells. The results indicated that antibody activities recorded in mixed haemadsorption on kidney cells could be effectively removed by absorption with both A and B erythrocytes. Absorptions with O erythrocytes were generally unsuccessful. After absorptions with rat erythrocytes the sera

could be divided into two groups; sera which were depleted of mixed haemadsorption activity and those which were very little affected by this absorption procedure.

There was no demonstrable correlation between high anti-kidney IgG antibody titres in sera from kidney recipients and rejection episodes.

DISCUSSION

A rise in heterophile antibodies, particularly to rat erythrocytes, was reported after renal transplantation in man (Rapaport *et al.*, 1967) and dogs (Almgård & Svehag, 1968). In these studies peak antibody titres tended to occur in association with acute rejection episodes. In more recent investigations by Kano & Milgrom (1970), Tiong & Morris (1972a) and McDonald *et al.* (1971) elevation of heterophile antibodies in kidney transplanted patients was confirmed. Although the antibody rise often preceded acute rejection, the conclusions concerning the relationship were conflicting.

In the present study significantly increased heterophile antibody titres were observed in twelve of twenty-seven patients after transplantation when multiple serum samples were taken over periods of several weeks. In seven of these twelve patients the titre rise appeared in close association with rejections. From these data no obligatory relationship between rises in heterophile antibodies and rejection can be claimed. It would seem however that patients with very high antibody titres ($\geqq$600) are prone to rejection (five out of five patients in Fig. 6). In a population of seventy-two apparently healthy blood donors only 5% had this high heterophile antibody titres.

Not immunosuppressed dogs receiving allogeneic kidney transplants showed distinct heterophile antibody responses at rejection or shortly thereafter. In addition, injection of washed allogeneic kidney cells induced formation of dog heterophile antibodies to rat erythrocytes at high titre (Almgård & Svehag, 1968). In analogy, release of kidney isoantigens shortly after transplantation or at times of impaired transplant function may be expected to stimulate such heterophile antibody formation also in human recipients. Due primarily to the variable and intensive immunosuppressive treatment these inducing events are apparently more difficult to register in the human situation. It is noteworthy that Rapaport *et al.* (1968) observed more potent and clearcut rises of heterophile agglutinins in not immunosuppressed human recipients of skin allografts than in kidney transplanted patients undergoing immunosuppressive treatment. But the different routes of antigen release and the uremic status of the kidney recipients may also have contributed to this difference.

The genesis and specificity of the heterophile antibodies to rat erythrocytes is still to large extent unclear. The titre rise after transplantation could not be explained on the basis of HL-A incompatibility (Tiong & Morris, 1972a; Kano & Milgrom, 1970). Other authors (Eichner *et al.*, 1963; Laskov *et al.*, 1968) reported that naturally occurring agglutinins to rabbit, dog and guinea-pig erythrocytes were related to the ABO blood groups. Tiong & Morris (1972a) presented substantial evidence indicating cross-reactivity between human A and B substance and rat erythrocyte antigens while Rapaport *et al.* (1967) failed to observe any association between ABO incompatibility and rises in heterophile antibody titres. Neither could these authors (Rapaport *et al.*, 1968) absorb the heterophile agglutinins with A or B erythrocytes. These two bodies of data would at first look seem difficult to reconcile.

From the present data we would like to conclude that:

(1) The pattern of reactivities exhibited by the recipient sera against rat, guinea-pig, sheep

and ox erythrocytes indicated that the heterophile agglutinins were neither directed against Forssman antigens nor were they Paul-Bunnel like antibodies.

(2) Increased titres of heterophile antibodies following kidney transplantation were not related to ABO-incompatibility as only ABO-compatible kidneys were used and titre rise occurred independent of the number of blood transfusions received.

(3) The presence of preformed lymphocytotoxic antibodies of broad anti-HL-A reactivity and high titres in two patients was not accompanied by any increased heterophile antibody level.

(4) Heterophile antibodies in transplant recipient sera could as a rule be rather effectively removed by absorption with allogeneic human kidney cells. Absorption with A or more frequently B erythrocytes abolished the heterophile activity in certain patient sera while absorption with O erythrocytes had no significant effect. Results of simultaneous titrations of sera from normal blood donors against rat erythrocytes and human A and B erythrocytes were in accordance with this observation.

(5) Part of the heterophile antibody activity had anti-B specificity as revealed by absorption tests and supported by the fact that rat erythrocytes reacted strongly in immunofluorescence with human anti-B serum. It is interesting to note that Gram-negative bacteria, and especially *E. coli*, seem to give rise to the formation of anti-B antibodies preferentially, when fed to healthy human individuals (Springer, 1970).

(6) The different absorbing capacity of A and B erythrocytes as compared to rat erythrocytes on antibodies detected by mixed haemadsorption on kidney cells was in agreement with the conclusion that sera with heterophile antibodies were heterogeneous with regard to antibody specificity. Thus, antibodies in one group of sera were effectively absorbed by allogeneic kidney cells, A or B erythrocytes and rat erythrocytes while the antibodies in another group of transplant recipient sera were absorbed only by kidney cells (known to contain A or B substance) and A or B cells.

In an attempt to explain the genesis of the heterophile antibody responses in transplant recipients' sera we propose that they are directed against B− substance related antigen in Gram-negative bacteria as well as against non-HL-A isoantigens in kidney and skin. This conclusion does not contradict the results of Kano & Milgrom (1970) and Tiong & Morris (1972b). For specificity or affinity reasons a subpopulation of these heterophile antibodies reacts with both rat erythrocytes, human B erythrocytes and allogeneic kidney cells while another population reacts primarily with rat erythrocytes and kidney cells.

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