

# Influence of Duration of Homograft on Humoral Responses in Man

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## **Introduction.**

Elevated titers of natural antibodies have been demonstrated in the sera of patients following transplantation.<sup>1-6</sup> These humoral responses were thought to be associated with rejection.<sup>7-10</sup> This concept was challenged by investigators whose studies associated these responses with infection or with injection of heterologous serum.<sup>11-15</sup> Nevertheless, the possible prognostic significance of these relatively simple tests has continued to evoke interest.

This study was undertaken to compare the humoral responses in three different groups of patients with organ transplants in order to evaluate the influence of duration of the homograft and the attendant immunosuppression.

## **Materials and Methods.**

### *Patient Selection.*

Fifty-eight patients who had received homografts were selected for study. The patients were divided into three groups: Group 1, 20 patients tested prior to transplantation and in the immediate post-transplant period with a mean duration of follow-up of 3.3 months; Group 2, 19 patients tested approximately five to seven years following transplantation with a mean follow-up duration of 82.2 months; and Group 3, 19 patients tested approximately ten years following transplantation with a mean follow-up duration of 118.1 months.

### *Tests for Anti-Rat Erythrocyte Antibodies (Heterophil Antibodies).*

Rat erythrocytes were washed three times in saline and reconstituted to a 2% suspension. The test sera were tested at a 1:20 dilution and in serial two-fold dilution for antibody titer. The tubes were allowed to stand at room temperature for 30 minutes, spun for 1 minute at 77 g, and read with the naked eye.

### *Tests for Rheumatoid Factor (Anti-Fc IgG antiglobulins).*

The sensitized human cell test (SHC) was used. A selected DCE/DCE test cell was sensitized with Ripley serum (high-titered antiDC) diluted 1:10. The Rh-positive cells were sensitized for 60 minutes at 37 C and then washed three times in saline. Test sera were titrated in saline in 0.1 ml volumes. Titers of 1:20 or above were considered positive.

### *Tests for Chymotrypsin Agglutinators (Anti-Fab IgG Antiglobulins) Hydrolysis of IgG globulins.*

The IgG globulins of anti-Rh serum (Ripley) were isolated by  $(\text{NH}_4)_2\text{SO}_4$  precipitation followed by chromatography on diethylaminoethyl (DEAE)-cellulose as previously described.<sup>16</sup> Methods of hydrolysis of the IgG globulins with chymotrypsin have been reported.<sup>17</sup>

*Sensitizations.* One milliliter (containing 5 mg of digested globulin) was added to 0.1 ml of human O, DCE/DCE washed packed cells and sensitized at 37 C for one hour. The sensitized cells were washed three times with saline and reconstituted to a 2% suspension. The cells were tested with goat anti-Fc and anti-Fab (Hyland Laboratories, Los Angeles, California) antisera.

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*Methods of testing.* Tests for serum agglutinators were performed in tubes by adding 0.1 ml aliquots of the sensitized cells to an equal volume of undiluted and serial twofold dilutions of sera to be tested. The mixture was allowed to stand at room temperature for ten minutes, and then spun for one minute at 77 g and read with the naked eye.

*Erythrocyte Sedimentation Rate (ESR).*

The Westergren method was used to document the ESR. The fall of the erythrocytes in millimeters in one hour was noted. The normal sedimentation rate with this method is 0 to 15 mm for men with an average of 4 mm, and 0 to 20 mm for women with an average of 5 mm.

*Nitroblue-Tetrazolium Test (NBT).*

The NBT test was performed according to the modified method of Dorwick<sup>18</sup> on venous blood collected in 4.5 ml vacutainers containing 3.8% sodium citrate. The NBT test was performed as soon as possible after the blood was drawn in order to eliminate morphological changes caused by prolonged exposure to the anticoagulant.

A standard solution of 0.4% NBT was prepared in a 10 ml stoppered volumetric flask by dissolving 20 mg of nitroblue tetrazolium (Grade III reagent, N6876, Sigma Chemical Co., St. Louis, Missouri) in 5 ml of sterile 0.85% isotonic saline. To dissolve the NBT, the stoppered flask was swirled under hot, running tap water until the solution became clear. This solution was then filtered and transferred to a brown bottle and stored at 4°C. The solution was stable for 72 hours.

Citrated blood, 0.2 ml, was placed in a test tube and 0.1 ml of 0.4% NBT was added. The tube was mixed by gently shaking, capped and incubated in a water bath at 37 C for 30 minutes. The tube was removed from the water bath, and one drop of the blood-NBT test mixture was added to two pre-cleaned, labeled glass slides. Special care was taken to avoid damage to the white blood cells. The slides were air-dried and stained with Wright's stain. The stained slides were examined microscopically using an oil immersion lens and 100 neutrophils were counted. Neutrophils were classified as "NBT-positive" if they contained any visible formazan deposits. Cells that were disrupted or clumped together were not counted. The percent of NBT-positive neutrophils was reported as 0% to 15% normal and 16% to 100% normal.

*Serum Electrophoresis.*

Serum electrophoresis was performed on cellulose acetate strips, using the Microzone cell, Model

R-101 (Beckman Instruments, Inc., Fullerton, California).

**Results.**

Figure 1 shows the humoral responses of the 20 patients in Group I during the first five to six months following transplantation. The anti-Fab antiglobulins are designated by dark circles. These antibodies are usually produced as IgG globulins. Only 7 of the 20 patients showed titers at any time in excess of the pretransplant values. Titers below 1:20 are shown as lines without a dot dipping below the 1:20 titer level. Fluctuations in titer are common.

The anti-rat erythrocyte antibodies are designated by open circles. These antibodies are almost always produced as IgM globulins. Fifty percent (10 out of 20) of the patients showed titers in the post-transplant period higher than the pretransplant titer. Once again, fluctuations in titer are common. There

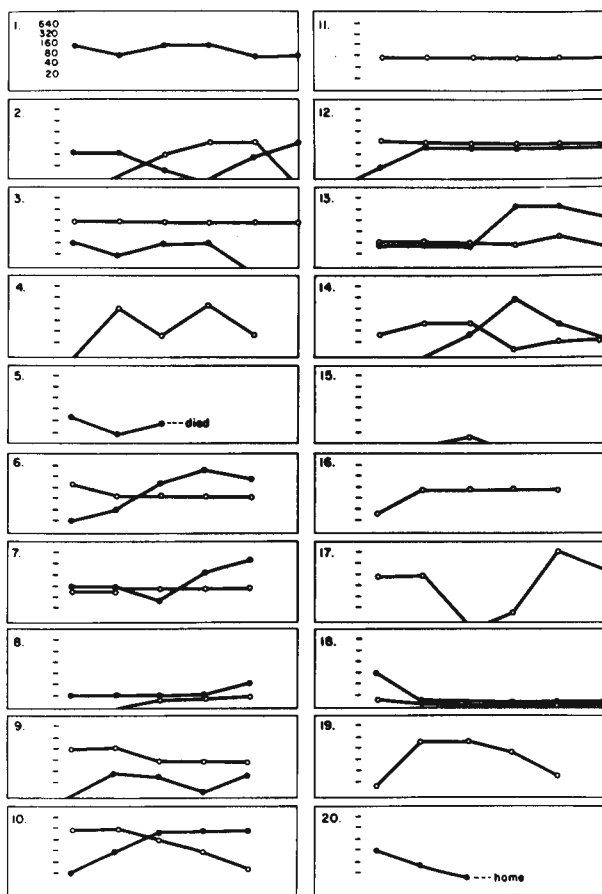


Fig 1—Titers of anti-rat erythrocyte antibodies and chymotrypsin agglutinators in 20 patients in the immediate post-transplant period. Open circle: anti-rat erythrocyte; Dark circle: chymotrypsin agglutinators.

is a tendency for both antibodies to return toward pretransplant levels 9 to 12 months after transplantation.

Although 4 of these 20 patients gave positive tests for rheumatoid factors, only 3 (patients 2, 7 and 13) showed titers in excess of the pretransplant values. Titers were not constant, as is observed in chronic rheumatoid arthritis, but fluctuated, cresting and then falling, like the other antibodies. The rheumatoid factors were invariably made as IgM globulins.

Modestly elevated titers of anti-sheep erythrocyte antibodies (1:80) were found in only 2 (patients 3 and 11) of the 20 patients.

The blood groups of the 58 patients did not significantly influence the serological responses of the antibodies tested.

Figure 2 shows the titers of the anti-rat erythrocyte antibodies (IgM globulins) and the anti-Fab antiglobulins (IgG globulins) in the three groups of patients in order of increasing duration of the homograft and attendant immunosuppression. The titers of the antibodies in Group 1 were read at three months post-transplant. The titers of Groups 2 and 3 were performed when they appeared for post-transplant check-up. It is apparent from Figure 2 that the number of patients with significant titers of antibodies decreases with duration of the homograft.

Three of the 38 long-term homograft recipients (five to ten years) had positive tests for rheumatoid factors. Neither the presence nor the titers of the rheumatoid factors were related to the duration of the graft. One patient who had shown high titers of rheumatoid factors in the past lost this activity 11 years post-transplant. This patient has developed reticulum cell sarcoma.

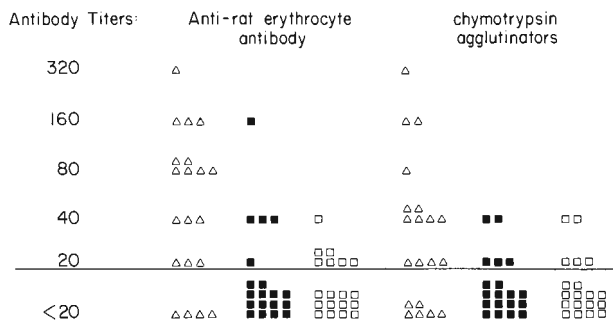


Fig 2—Influence of duration of homograft and attendant immunosuppression on humoral antibodies in 58 patients. *Triangle*: mean 3.3 months; *Dark square*: mean 82.2 months; *Open square*: 118.1 months.

*Relationship of level of gamma globulin to the production of humoral antibody.*

Electrophoretic patterns of the serum proteins were performed on the three groups of patients. The patients were divided into two groups, those with gamma globulins below 12% and those with gamma globulins above 12% (Table 1). In Group 1, 5 out of the 6 patients with gamma globulin levels below 12% made anti-Fab IgG antibodies as did 9 out of the 14 with gamma globulin levels above 12%. Thus, 70% of Group 1 made these antibodies. However, in Group 2, only 2 out of 11 made these antibodies when the level of gamma globulin was below 12%, while 3 out of 8 made them when the level of gamma globulin was above 12%. In the whole group, 26% made these antibodies. In Group 3, none of the 6 patients with gamma globulin levels below 12% made these IgG antibodies, while 5 of the 13 with gamma globulin levels above 12% did so.

A similar pattern is seen for the IgM anti-rat erythrocyte antibodies. There appears to be no effect of low levels of gamma globulin in the immediate post-transplant group (Group 1), but in the long-term patients, the influence of low levels of gamma globulin on both the IgG and IgM antibodies is demonstrable.

Table 2 shows the relationship between the serum creatinine levels and the failure to show humoral responses in 37 of the long-term transplant recipients. Among the 12 patients with elevated levels of creatinine, the incidence of both IgG and IgM antibodies is decreased. This decrease in antibody production was

a) Relationship of level of gamma globulins to IgG antibody production as chymotrypsin agglutinator			
	<12%	>12%	Total
Group 1*	5/6	9/14	14/20 (70%)
Group 2**	2/11	3/8	5/19 (26%)
Group 3***	0/6	5/13	5/19 (26%)

b) Relationship of level of gamma globulins to IgM antibody production as anti-rat antibody			
	<12%	>12%	Total
Group 1*	6/6	10/14	16/20 (80%)
Group 2**	2/11	3/8	5/19 (26%)
Group 3***	1/6	6/13	7/19 (37%)

\* Mean 3.3 months  
 \*\* Mean 82.2 months  
 \*\*\* Mean 118.1 months

TABLE 2

Thirty-seven patients with long-term homografts showing a relationship between the serum creatinine level and failure to show humoral responses

		IgM Antibody Responses (anti-rat erythrocyte antibody)			
Creatinine levels:	3.1 and above 0/3	2.6-3.0 0/1	2.1-2.5 0/2	1.5-2.0 1/6	0.9-1.4 11/25
		IgG Antibody Responses (chymotrypsin agglutinators)			
Creatinine levels:	3.1 and above 0/3	2.7-3.0 0/1	2.1-2.5 0/2	1.5-2.0 0/6	0.9-1.4 10/25

not related to a significantly higher dose of maintenance immunosuppression.

Since infection plays a prominent role in patients maintained for long periods on immunosuppressive agents, the erythrocyte sedimentation rate and the NBT test were performed on 37 of the 38 patients in Group 2 and 3. The erythrocyte sedimentation test was abnormal in 24 of the 37 patients (65%). The numerous factors affecting this test, such as anemia and dysglobulinemia, lessen the value of this test for evaluation of patients with positive tests. The NBT test was only positive in 3 of the 37 long-term patients. Careful review of the discharge summaries corresponding to the period at which the test was done failed to reveal a reason for these abnormal tests. In a further effort to evaluate the significance of these positive tests, C3 and C4 levels were determined on these patients' sera along with 12 other patients who did not show positive NBT tests. The levels of C3 and C4 were not significantly altered in the patients with positive NBT tests.

#### Discussion.

These studies document humoral antibody activity in the immediate post-transplant period. Rises in titer of both IgG antibodies (anti-Fab IgG antiglobulins) and IgM antibodies (anti-rat erythrocyte antibodies) are demonstrable. The antibody rises are usually inverse to each other, implying that different antigens elicit these responses. The antigens on the rat erythrocyte are also found on dog and rabbit erythrocytes, and related antigens are present on human B erythrocytes. Thus, the antibodies are termed heterophils. McDonald and co-workers<sup>19</sup> recently studied these antibodies and related the absence of the "rat" antigen (HT-A) in the recipients to increased rejection of kidneys from donors with the antigen. Their studies have clinical importance and help to define complex tissue antigens which will receive more attention in the future.

Rises in titer of the anti-Fab IgG antiglobulins

could not be closely associated with rejection. These antiglobulins are associated with severe suppurative infection in a hospitalized population.<sup>16</sup> In post-transplant patients, there is a tendency for these antiglobulins to rise when the titers of the anti-rat erythrocyte antibodies are falling,<sup>14</sup> which suggests that these are auxiliary immune responses or responses to antibody-antigen complexes.

The dramatic serological responses in the immediate post-transplant period are not repeated in subsequent years. This is probably due to immunosuppressive therapy. However, we observed no inhibition of polymorphonuclear phagocytosis nor depression of C3 or C4 levels. On the other hand, laboratory tests for infection (sedimentation rate, NBT test) may react nonspecifically, making the diagnosis of occult infection difficult.

As all those interested in transplantation know, the greatest drawback in currently available methods of immunosuppression is their nonspecificity. This fact has stimulated interest in immunological enhancement of the kidney graft. Evidence exists that kidney grafts in rats can survive by virtue of enhancement.<sup>20-25</sup> One approach in humans entailed the production of immune sera to leukocyte antigens, followed by the hydrolysis of the antibody to remove the complement-fixing Fc fragment. The resulting Fab fragments were injected into the recipient to bind to the antigenic sites on the graft.<sup>26</sup> As discussed previously, the serum agglutinators are antiglobulins which have specificity for Fab fragments following hydrolysis. These antiglobulins are naturally occurring IgG globulins commonly found in low titer. However, the serum agglutinators (named homoreactant in rabbits) are not apparently stimulated by the injection of Fab fragments,<sup>27</sup> but once stimulated, their ability to reconstitute the biological potential of the Fab fragment has been established.<sup>28</sup> Therefore, it would be essential to test the recipient for these antiglobulins prior to the injection of the Fab fragments.

In any case, the role of the anti-Fab antiglobulins must be considered when Fab fragments are used to achieve blocking of the antigenic sites of a homograft.

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