# *WATER-SOLUBLE HUMAN TRANSPLANTATION ANTIGEN**

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Although there has been considerable study of the chemistry of the antigens of rodents,[1, 2] the nature of human transplantation antigens has received relatively little attention.[3] Recent progress in the understanding of rodent transplantation antigens has been afforded by exposure of dissociated cells to low-intensity ultrasound in order to solubilize these materials from the cellular membranes. The present studies were undertaken to determine whether this method of solubilization and isolation is applicable to the human transplantation antigens of the HL-A system.[4-6]

*Materials and Methods.—Procurement of organs:* Spleens were obtained from five donors in the Northern Italian area who had been splenectomized for the following indications: LAV, Cooley's anemia in a 3-year-old boy; BRA, a hemorrhagic cyst attached to the spleen of a 35-year-old women; NAC and PAT, two 35-year-old women with Banti's syndrome; and PER, gastric carcinoma in a 65-year-old woman. The HL-A phenotypes of the donors are summarized in Table 1.

*Preparation of the antigen:* The method of preparing transplantation antigen has previously been described in detail.[2] Aliquots (50-ml) of the cell suspension of 30–40 million cells/ml were exposed to a 3–5-min burst of 15.5 w/cm$^2$ sonic energy generated over a diaphragm 4.7 cm in diameter in a Raytheon model DF 101 magnetostrictive oscillator. The sonicated suspension was first centrifuged to remove the cellular membranes. The ultracentrifugal supernate was concentrated against Aquacide I, Grade C (Calbiochem) and then chromatographed in ascending fashion on Sephadex G-200 (Pharmacia). The fraction (Sephadex fraction 1) which appeared at the front of the inner volume was concentrated against 50% sucrose (enzymatic grade) and then dialyzed against 0.9% sodium chloride buffered at pH 7.4. Concentrated Sephadex fraction 1 was applied to discontinuous polyacrylamide gels prepared according to the pH 9.4 system previously described.[2] Aliquots of Sephadex fraction 1 were labeled with $^{125}$I according to the method of McFarlane,[7] and excess label was removed by passage over Sephadex G-10. After electrophoresis and slicing, the radioactivity patterns of the gel cuts were determined in a gamma-scintillation apparatus. Protein concentrations were estimated by the Lowry method[8] with Lab Trol reagent as standard.

*In vivo tests:* Intradermal challenge with antigen was performed in individuals who had been previously immunized either by planned transfusions or by the intramuscular administration of leukocytes[9] and who were the donors of typing antisera. These immunized subjects, spleen donors, and nonimmunized individuals from the same population group were given duplicate intradermal injections of antigen (10 $\mu$g of Sephadex fraction 1 or 0.1 $\mu$g of acrylamide components) and of buffer on the volar surface of the forearm. After the administration of antigen, there was never any indication of temperature elevation, pruritus, necrosis, or systemic reaction to the materials; and only rarely did the individual complain of local pain. Skin test sites were read at 6, 24, 48, 72, and 96 hr after injection for the dimensions and depth of erythema, for the presence of hemorrhage or central discoloration, and for the extent of induration and the degree of skin thickening. Areas of the reactions were calculated in millimeters as the mean radius squared.[2] Some patients were also injected intradermally with 100 $\mu$g of tuberculin.

*In vitro tests:* The microdroplet cytotoxicity test of Terasaki and McClelland[10] was employed in the manner previously described.[9] The test sera and their specificities in-

TABLE 1. *Tissue types of the spleen donors and of the test lymphocytes for the seven HL-A specificities examined.*

| Specificity: | 5 | 5 + X | 11 | 6 | 7 | 8 | 8 + X | 9 | 10 | 12 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Serum:* | 399 | 4.16 | 36.05 | 400 | 10.32 | 32.19 | 16.18 | 11.34 | Storm | 38.06 | 35.10 |
| Donors | | | | | | | | | | | |
| LAV | − | − | − | + | − | + | + | − | − | + | + |
| BRA | + | + | − | − | − | + | + | − | − | − | − |
| NAV | + | + | − | + | + | + | + | + | (−) | − | − |
| PAT | − | − | + | + | − | − | − | − | − | + | + |
| PER | − | − | + | + | + | − | − | − | − | − | − |
| Lymphocytes | | | | | | | | | | | |
| MAT | − | − | − | 64† | 32 | 32 | 64 | − | − | 16 | 16 |
| CAS | − | − | − | − | − | 32 | >128 | − | − | 32 | 16 |
| CUR | 16 | 8 | − | 64 | 16 | − | − | 16 | − | − | − |
| MAS | − | − | 64 | 16 | − | − | − | − | 16 | − | − |

* The sera bear the same designations as described and used by Ceppellini *et al.*[9]
† Reciprocal of titer of each positive serum against each set of test cells.

cluded TO 5 (absorption 399), TO 5 + X (4.16), TO 11 (36.05), TO 6 (absorption 400), TO 7 (10.32), TO 8 (32.19), TO 8 + X (16.18), TO 9 (11.34), TO 12 (38.06), and TO 12 (35.10); all of which were raised and characterized by Ceppellini *et al.*[6] A serum against TO 10 (Storm) was obtained from Walford through the NIAID Serum Bank. Except as noted for 4.16 and for 16.18, all the sera are monospecific. Each serum was tested against the separated peripheral leukocytes of four donors. The lower half of Table 1 shows the reciprocal of the titer of each serum against each set of target cells.

Inhibition experiments were performed by incubating (for 60 min at 25°C) duplicate sets of four serial dilutions of antigen with two dilutions of each test serum. The sera were employed at two and at four cytotoxicity units; for example, MAT cells, 95% of which are killed by test serum 400 diluted 1:64, were used as target cells for antisera which had been preincubated at dilutions of 1:16 (4 units) and 1:32 (2 units). Antigens were considered to have effected significant inhibition if the cytoxic activity of the antiserum had been decreased by more than 60% in tests performed in duplicate and employing three sets of target cells.

*Results.—In vivo tests with Sephadex fraction 1:* The Sephadex fraction 1 antigen (10 μg) obtained from LAV, BRA, NAV, and PAT was administered intradermally to 12 "normal" volunteers and to one spleen donor. The donor PAT showed no reaction to her own antigen. Nine of the normal individuals developed only modest erythema which rapidly declined after four to six hours and was barely discernible at 24 hours. However, two normal men and the only multiparous woman on the panel developed reactions with strong erythema and moderate induration at 24 hours. It is noteworthy that the HL-A phenotypes of the two men who displayed strong reactions to 10 μg of NAV antigen showed more antigenic differences from the donor NAV than did the phenotypes of the two individuals who failed to react against the antigen (Table 2).

In contrast to these controls, all five persons who had been preimmunized and were producing antibodies directed against antigenic specificities present in the donor antigen displayed well-defined, deep erythematous reactions with a violaceous hue and central discoloration. For example, the antigen of BRA elicited a strong reaction in the patient QUE who produces anti-TO 8 antibody, but not in the patient DUC who produces anti-TO 6,7 antibody. Furthermore, antigen prepared from PAT elicited violaceous reactions in BUF, who produces

TABLE 2. *HL-A phenotypes of normal individuals injected with NAV Sephadex fraction I.*

| Name | Specificity 2 | 3 | 4 | 5 | 11 | 6 | 7 | 20 | 8 | 9 | 10 | 12 | 13 | Differences (no.)* | Reaction† ($mm^2$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Donor NAV | + | + | + | + | − | + | + | − | + | + | − | − | − | | |
| Recipient ZAN | + | + | − | − | − | + | − | − | + | − | − | + | − | 4 | 16 |
| Recipient MART | + | + | − | − | − | + | − | − | − | + | − | − | + | 4 | 19 |
| Recipient MARC | + | + | − | − | − | + | − | − | − | − | − | − | − | 5 | 77 |
| Recipient POR | − | − | − | − | − | + | + | + | − | − | + | − | − | 6 | 85 |

* Specificities present in antigen donor and absent in recipient.
† Reactions as average radius² in $mm^2$.

anti-TO 2,4,5 antibody, and in PER, who produces anti-TO 6,7 antibody, but not in FRA with anti-TO 9 or in CAR with anti-TO 5 antibody. In contrast to control sites where tuberculin had been injected, the antigen test sites of immunized persons showed a more rapid progression of events, a hemorrhagic violaceous hue, and central discoloration, all stigmata indicative of an Arthus reaction.

*In vitro tests with Sephadex fraction 1:* Table 3 summarizes all the results of duplicate inhibition tests employing five antigenic preparations with eleven antisera.[11] In general, these results conform with the predictions from tissue typing or from absorption studies: 38 of 43 tests are in agreement. For example, the antigen of LAV inhibited both of the anti-TO 12 sera, but failed to inhibit an anti-TO 5 or an anti-TO 9 serum. Similarly, the antigen of BRA inhibited two anti-TO 5 sera, but not an anti-TO 11, an anti-TO 6, an anti-TO 7, an anti-TO 9, or one of the anti-TO 12 sera. The phenotype of the donor NAV is rather unusual; however, it has been confirmed by two sets of absorptions. The antigen of NAV inhibited the cytotoxic activity of an anti-TO 5, an anti-TO 6, an anti-TO 7, and an anti-TO 8 serum, but not an anti-TO 11 or an anti-TO 12 serum. The failure of NAV antigen to inhibit serum 4.16 which is directed against TO 5 + X or serum 16.18 against TO 8 + X is probably due to the absence of factor X in the antigenic preparation. Thus, although the antigen may have absorbed the antibody against TO 5 or TO 8, it did not interfere with the action of antibody against factor X, which was present on the target cells. This finding accentuates the need for operationally monospecific sera in the strictest sense of Walford's definition.[12]

Three types of unpredicted reactions were observed. (1) There was the inhibition of the TO 7 antiserum by LAV antigen. Due to the overlapping distribution of the TO 6 and TO 7 specificities, it is possible that LAV is indeed TO + 7 and that serum 10.32 exhibited a cytotoxicity-negative-absorption-positive (CYNAP) reaction, as had been previously seen with the 10.37 serum of the $4^b$ series.[13] In order to identify this phenomenon, the HL-A phenotypes of BRA, NAV, PAT, and PER were determined not only by the cytotoxic action of typing antisera on donor cells, but also by the capacity of donor cells to absorb the activity of the antisera. Seven instances of CYNAP reactions were documented

TABLE 3. *Inhibition of the cytotoxic reactions of typing antisera by Sephadex fraction I.*

| Specificity: | 5 | 5 + X | 11 | 6 | 7 | 8 | 8 + X | 9 | 10 | 12 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Serum: | 399 | 4.16 | 36.05 | 400 | 10.32 | 32.19 | 16.18 | 11.34 | Storm | 38.06 | 35.10 |
| Donor LAV | | | | | | | | | | | |
| Phenotype[c] | − | − | − | + | − | + | + | − | − | + | + |
| Antigen | − | ND | ND | ND | 0.008[a] | ND[b] | ND | − | ND | 0.008 | 0.004 |
| Donor BRA | | | | | | | | | | | |
| Phenotype | + | + | − | − | − | + | + | − | − | − | − |
| Absorption | + | + | − | − | − | + | + | − | + | − | + |
| Antigen | 0.01 | 0.015 | − | − | − | −[a] | − | − | 0.005 | − | 0.01 |
| Donor NAV | | | | | | | | | | | |
| Phenotype | + | + | − | + | + | + | + | + | (−) | − | − |
| Absorption | + | + | − | + | + | + | − | + | + | − | − |
| Antigen | <0.003 | − | − | 0.003 | <0.003 | 0.003 | − | −[a] | <0.003 | − | − |
| Donor PAT | | | | | | | | | | | |
| Phenotype | − | − | + | + | − | − | − | − | − | + | + |
| Absorption | − | − | + | + | − | − | − | − | + | + | + |
| Antigen | − | − | 0.005 | 0.005 | − | 0.005[a] | − | − | 0.005 | −[a] | 0.005 |
| Donor PER | | | | | | | | | | | |
| Phenotype | − | − | + | + | + | − | − | − | − | − | − |
| Absorption | ND | ND | ND | + | + | ND | ND | + | ND | + | + |
| Antigen | ND | ND | ND | 0.006 | 0.012 | ND | ND | 0.025 | ND | 0.05 | 0.006 |

[a] Inhibitory pattern inconsistent with tissue typing.
[b] These reactions were not performed.
[c] Phenotype determined by cytotoxicity reactions.

in the present study. Three instances were noted with the serum Storm anti-TO 10. Since no individual was examined whose cells failed to absorb this serum, it may be highly susceptible to inhibition, and thus of limited utility in studies of this type. CYNAP reactions were observed twice with the anti-TO 12 serum 35.10, once with the anti-TO 12 serum 38.06, and once with the anti-TO 9 serum 11.34.

(2) Three instances of typing-positive-absorption-positive-antigen-negative (TAPAN) reactions were noted: BRA antigen with serum 32.19, NAV antigen with serum 11.34, and PAT antigen with serum 38.06. Insight into this phenomenon was gained in the instance of BRA antigen: $5 \times 10^{-4}$ $\mu$g of acrylamide-purified BRA active component obtained from this Sephadex fraction 1 inhibited the cytotoxic action of antiserum 32.19. Therefore, the inability of the crude Sephadex fraction 1 to inhibit this antiserum may have been due to either an insufficient quantity of antigen or to an interfering contaminant substance.

(3) One typing-negative-absorption-negative-antigen-positive (TANAP) reaction was observed: PAT antigen inhibited the anti-TO 8 serum 32.19. This type of reaction pattern is more difficult to explain in the context of our present knowledge of the antigenic determinants of the HL-A system.

*Biologic and serologic tests of acrylamide fractions:* The crude Sephadex fractions were subjected to discontinuous electrophoresis on $7^1/_2$ per cent polyacrylamide gels at pH 9.4. After electrophoresis of $^{125}$I-labeled LAV antigen, the radioactivity of each of 40 slices was determined, revealing six major components. Four other acrylamide gels onto which unlabeled LAV fraction 1 had been applied were divided into five equal zones. Three preimmunized and one normal individual were injected intradermally with the eluate of each zone of a single gel and with the eluate of a zone from a control gel (Fig. 1). The normal person VAS did not show a reaction to the antigen. On the other hand, the preimmunized individuals displayed strong reactions with maximum biological activity localized in a fast-moving component which ran between zones IV and V ($R_F$ about 0.80). The gel slices of zones IV and V were combined after elution and electrophoresed again on another $7^1/_2$ per cent gel at pH 9.4. This second gel was cut into 40 slices, and each slice was eluted. The eluates of cuts 11 and 12 elicited the strongest direct cutaneous reactions. As a control, the eluates of cuts 10–15

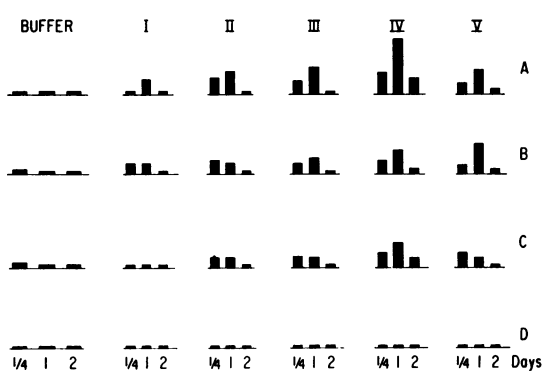


FIG. 1.—Columns depicting the reaction sizes (in mm$^2$) of cutaneous reactions. (*A*), (*B*), (*C*) The three preimmunized individuals who received electrophoretic zones I, II, III, IV, and V, and buffer; (*D*) the reactions elicited by zones, I, II, III, IV, and V, and by buffer in a normal individual.

from a $7^1/_2$ per cent gel on which BRA Sephadex fraction 1 had been electrophoresed produced no reaction when injected into the donor BRA.

Gel eluates from the spleen of NAV were administered to two normal men and to four individuals who had been preimmunized with $60 \times 10^6$ donor spleen cells divided between three intradermal sites. The evolution of the reactions at these sites was examined over a seven-day period. Two individuals (BIA and SGH) who showed the strongest responses to the immunizing stimulus were injected with two sets of acrylamide cuts: one set obtained from Sephadex fraction 1, and the other a negative control preparation. The other two immunized (CAP and CRE) and the two nonimmunized (ROC and BOT) individuals received only one set of eluates—those from Sephadex fraction 1. ROC and BOT had transitory erythema which disappeared almost entirely by 24 hours. At 24 hours, BIA and SGH displayed strong cutaneous reactions of 144 $mm^2$ and 182 $mm^2$ to the eluates of fraction 1 cuts 9 and 10, but no reaction to the eluates from the other antigen. CAP and CRE had 42 $mm^2$ and no reactions to cuts 9 and 10, respectively (Fig. 2). A comparison of the phenotypes of the immunized individuals for 12 leukocyte factors of the HL-A system revealed that only CRE had the same $4^a$ phenotype as the donor. For the specificities TO 2–13, CAP differed from donor NAV at TO 5, 6, 7, and 9, CRE at TO 7, 8, and 9, BIA at TO 5, 7, and 8, and SGH at TO 5, 7, and 9.

In addition, aliquots of eluates from each cut of the LAV second gel were examined for inhibition of typing antisera. As predicted from the HL-A phenotype, the eluates of LAV cuts 10 and 11 ($R_F$ 0.78–0.80) inhibited the anti-TO 12 serum 35.10, but not the anti-TO 9 serum 11.34. Absorption of serum 35.10 with antigen reduced the number of killed target cells from 95 per cent to 22, 32, 36, and 44 per cent. The acrylamide gel eluates of cuts 11–15 from the spleen BRA also showed specific inhibitory activity: 5, 14, 16, 11, 10, 10, 16, 14, 5, and 5 per cent target cells killed after addition of antigen to the anti-TO serum 35.10, and 5, 5, 14, 5, 20, 16, 5, and 15 per cent after addition of antigen to the anti-TO 8 serum 32.19. On the other hand, there was no localized inhibition of the anti-TO 9 serum 11.34. The broad zone of inhibition corresponds to zone IV of the first LAV gel and reflects the high concentration of antigen applied to the gel (1 mg).

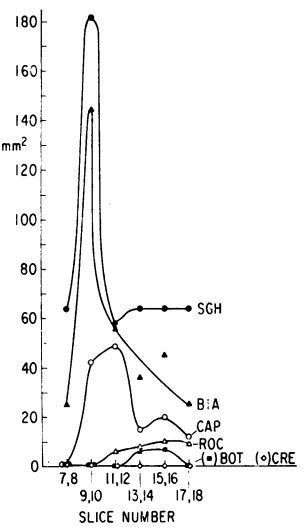


FIG. 2.—Cutaneous reactions of preimmunized individuals SGH, BIA, CAP, and CRE and of normal individuals BOT and CRE to acrylamide gel cuts 7 and 8, 9 and 10, 11 and 12, 13 and 14, 15 and 16, and 17 and 18 after electrophoresis of NAV Sephadex fraction 1 on a $7^1/_2$ % gel at pH 9.4.

*Isolation of component 3:* Re-electrophoresis of the active zone $R_F$ 0.78–0.80 disclosed two components. Separation of these components was obtained by decreasing the porosity of the gel and by performing the electrophoresis in the

presence of 8 *M* urea. The contaminant material was component 2 $R_F$ 0.83, which could not be resolved from component 3 on either a 7 1/2 or a 5 per cent gel, but could be separated on a 10 or a 15 per cent gel in the presence of urea. Studies using acrylamide fractions rich in components 2 or 3 revealed that the component retarded on the increased porosity gels was component 3. Component 3 was shown to be electrophoretically homogeneous since it re-electrophoresed as a single band sequentially on a 15 per cent gel in 8 *M* urea, on a 7 1/2 per cent gel without urea, and finally on a 15 per cent gel containing urea.

*Discussion.*—The results presented herein demonstrate that at least a portion of the HL-A transplantation antigen is liberated in a water-soluble state from splenic cells by a short, controlled burst of low-intensity sound. There was good agreement between the predictions from tissue typing, employing cytotoxic and absorption reactions, and the inhibitory patterns of the extracted transplantation antigens: eight antigenic specificities, including representatives of the $4^a$, the $4^b$, and LA series, were detected. In addition, the substance that inhibited the cytotoxic reactions and elicited Arthus-type cutaneous hypersensitivity reactions was present in the same band on gel electrophoresis; and this band was electrophoretically similar to the component known to accelerate the rejection of donor-type skin grafts and to elicit delayed-type hypersensitivity reactions in guinea pigs. This finding confirms the hypothesis of Brent *et al.*[12] that the same antigen induces both the humoral and the cellular responses in transplantation immunity. However, it is not known from the present work whether the very same determinants of this single component mediate these antigenic activities.

*Summary.*—Water-soluble human transplantation antigen was liberated from splenic cells by exposure to a short, controlled burst of low-intensity sound and was purified by gel filtration on Sephadex G-200 followed by discontinuous electrophoresis on polyacrylamide gels.

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[11] Extensive controls were introduced in order to assure the specificity of the inhibitory action of the antigen. (1) Varying amounts of Lab Trol gamma-globulin concentrate were added to antisera in order to determine whether proteins inhibited the cytotoxicity reaction. At concentrations of less than 1 $\mu g/\mu l$, this type of nonspecific activity was not observed. (2) The incubation of cells, antigenic preparations, and complement in the absence of antisera never revealed a cytotoxic effect of these antigens. (3) Only when the antigen was used in amounts greater than 0.1 $\mu g/\mu l$ were there cases of nonspecific inhibition. At these high protein concentrations, there frequently occurred complete inhibition of all antisera. Whether this effect was due to specific interference with the action of complement or to other factors in the cytotoxicity reaction is presently unknown. (4) The most important specificity control was the over-all design of the experiments: antigenic preparations from several donors were tested with a single set of sera at fixed titers and with four different sets of target cells. According to this design, anomalies in the behavior of the sera or of antigens were readily evident by the nonspecific trend of the data.

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