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Demonstration of Alloantibodies to HL-A Antigens by Immunofluorescence

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Summary. Antibodies to HL-A antigens on the surface of human peripheral blood lymphocytes have been demonstrated by the method of indirect immunofluorescence (IF). The utilization of IF for the detection of pre-existing humoral sensitization to donor alloantigens, particularly weakly reactive or non-complement fixing HL-A antibodies, in prospective allograft recipients is suggested.

Key words: Transplant recipient, presensitization, alloantibodies, immunofluorescence

The failure to detect pre-sensitization to donor alloantigens prior to transplantation in patients exhibiting a sudden onset of acute humoral renal allograft rejection suggests the occurrence of a secondary or anamnestic type of immunologic response by the recipient to alloantigens of the graft (7). Presently, definition of HL-A specificities are primarily dependent upon the ability of: 1) selected sera to lyse peripheral blood lymphocytes (PBL) in the presence of complement (C') or 2) platelets sensitized with antibody to fix C'. However, as all cells possessing surface antigens may not be equally sensitive to the lytic effect of antibodies and C' (5), and as a good proporation of HL-A antibodies are 7S immunoglobulins (Ig) and as such are rather inefficient in fixing C' (4), this may explain in part, the sometimes variable and disconcerting results obtained in attempting to detect pre-sensitization to donor alloantigens.

As the presence of genetically determined alloantigens may be readily identified on the surface of normal and neoplastic cells by immunofluorescence (IF, Ref. 6), it was thought that a similar application of IF to demonstrate alloantibodies to HL-A antigens might serve to augment present methods for the detection of pre-existing humoral sensitization to donor alloantigens in prospective recipients.

Portions of this report have appeared in abstract form (1).

Materials and Methods

IF staining of viable suspensions of $3\text{-}5\text{x}10^6$ PBL/ml in phosphate buffered saline pH 7.2 containing 0.1 per cent EDTA-Na_2 (PBS-EDTA) from individuals with known HL-A and erythrocyte (RBC) antigens was affected by incubation for 30 min. of equal volumes (0.05 ml) of washed cells and dilutions (made in PBS-EDTA) of heat-inactivated (56° C for 30 min) monospecific or polyspecific alloimmune anti-HL-A antisera (obtained from the NIH Serum Bank) and polyspecific anti-HL-A antisera obtained from polytransfused patients and multiparous women with known reactivity. PBL were then washed 2x in the diluent and resuspended for 30 min in 0.05 ml of a fluorescein (fluorescein isothiocyanate) labeled goat antiserum to human IgG (γ -chain). This fluorescent conjugate had an apparent F:P ratio of 1.44 and was diluted to 1/4 to 1/2 units of anti-IgG/ml (3) prior to use in PBS-EDTA. Following staining the cells were washed 2x in diluent, resuspended in PBS-EDTA, placed on microscope slides to air dry and mounted on coverslips possessing 95% glycerol. Preparations were then exaimed under oil emersion with UV illumination. Direct IF staining to permit distinction between IF staining

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due to binding of anti-HL-A and anti-IgG antibodies to HL-A and Ig antigenic determinants, respectively, was performed in the same manner, by excluding the first step. In cases where differentiation between membrane-bound HL-A antigens and Ig determinants was questionable, distinction was made by successive double labelling, i.e., PBL were first stained for Ig by incubation for 30 min in a rhodamine conjugated antiserum to human γ-globulin, washed 2x in PBS-EDTA and then stained for HL-A as described above.

In addition to the test serum, a saline control, as well as an alloimmune serum possessing antibodies corresponding to one or more of the HL-A antigens present on the target cell donor's PBL, designated as a homologous serum control, and an alloimmune serum lacking the corresponding HL-A antibodies to HL-A antigens present on the target cell donor's PBL or a serum, e.g., normal AB serum, devoid of HL-A antibody activity, designated as a heterologous serum control, were included in all tests.

Absorption of sera was performed by incubation of heat-inactivated dilutions of the test sera with autologous or isologous PBL possessing or lacking HL-A antigens corresponding to those anti-HL-A antibodies present at 37 C for 1 hr., and then overnight at 4° C.

Detailed methodology of cell preparation and IF staining shall appear in a subsequent publication (2).

Results

IF staining of PBL with homologous serum, i.e., a serum possessing HL-A antibodies corresponding to one or more of the HL-A antigens present on the target cell donor's PBL, e.g., PBL with HL-A antigens 2, 12, and 40 reacted with an anti-HL-A 12 antiserum, disclosed a discrete pattern of membranal IF (Arrow 1, Fig. 1). This HL-A type of IF staining could be distinguished from IF staining identifying membrane-bound Ig determinants on the surface of PBL in the same preparation (Arrow 2, Fig. 1). When required, resolution of questionable differentiation between the IF identification of HL-A antigens and Ig determinants was accomplished by double labelling as shown in Fig. 2. Similar IF staining of the same PBL with a heterologous serum, i.e., a serum devoid of HL-A antibodies or lacking the corresponding HL-A antibodies to the type of HL-A antigens present on the target cell donor's PBL, e.g., anti-HL-A 9, was not obtained as illustrated in Fig. 3, which shows IF staining of membrane-bound Ig only, as previously seen in Fig. 1, (Arrow 2) and in Fig. 2 (Arrows 2 and 3).

The IgG nature of IF staining reactions observed with anti-HL-A antisera was confirmed by incubation of PBL with unlabelled goat antiserum to human IgG prior to staining with the fluorescein conjugated antiserum.

Table 1 summarizes the results of the repre-

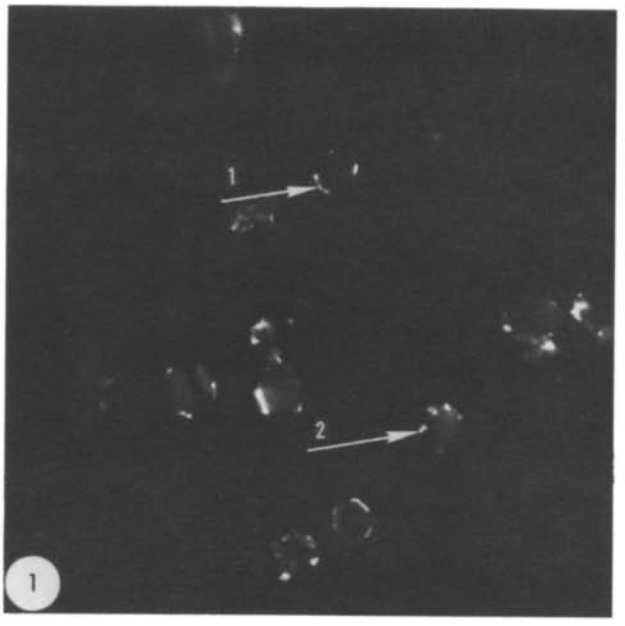
Table 1. Summary of Representative Series of Experiments Illustrating Identification and Specificity of HL-A Antibodies by Indirect Immunofluorescence (IF)

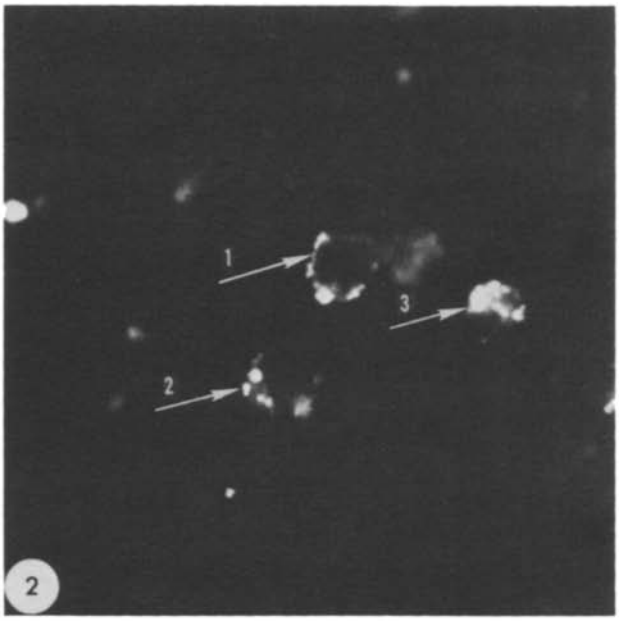
Specificity of Serum	Absorbed with PBL[a] with HL-A Antigens	IF staining for membrane-bound: HL-A-Antigens 2, 12, 40	HL-A-Antigens 2, 3, 7	Ig[a] Determinants
(Saline)	-	Negative[b]	Negative	Positive[b]
Anti-HL-A 9[c]	-	Negative	Negative	Positive
Anti-HL-A 12[c]	-	Positive	Negative	Positive
	2, 3, 7	Positive	Negative	Positive
	2, 12, 40	Negative	Negative	Positive

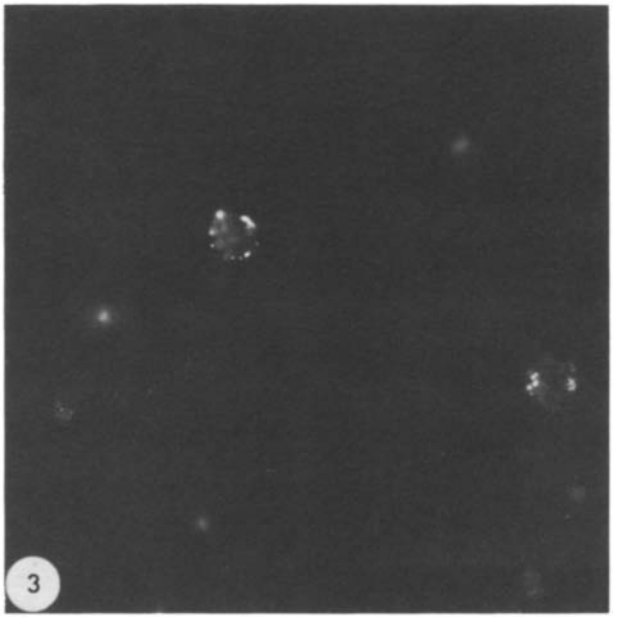
a PBL, human peripheral blood lymphocytes, Ig, immunoglobulin.

b Negative, indirect IF staining titre < 1:2, positive indirect IF staining titre ≥1:2.

c Anti-HL-A 9 and anti-HL-A 12 defined as heterologous and homologous sera, respectively, in the present study as based on the identification of HL-A antigens on the PBL with which they were reacted.







Figs. 1 to 3 are representative photomicrographs of indirect immunofluorescent (IF) staining reactions demonstrating the identification and specificity of HL-A antibodies on the surface of human peripheral blood lymphocytes (PBL).

Fig. 1.: PBL from individual with HL-A antigens 2, 12 and 40 incubated with monospecific anti-HL-A 12 antiserum followed by treatment with fluorescein labelled antiserum to human IgG. 1, identifies binding of HL-A antibodies to HL-A antigens. 2, indicates IF staining of immunoglobulin (Ig) determinant. x400 with oil

Fig. 2.: Illustrates distinction between IF staining of HL-A antigens (1) and Ig membrane-bound determinants (2 and 3) following successive double labelling with rhodamine conjugated antiserum to human γ-globulin, anti-HL-A antisera and fluorescein conjugate to human IgG. x640 with oil

Fig. 3.: Identical PBL as shown in Figure 1, following incubation with monospecific anti-HL-A 9 antiserum and treatment with fluorescein conjugate. Note presence of IF staining of membrane-bound Ig only. x 400 with oil

sentative experiments reported and further illustrates the specificity of these IF staining reactions following: 1) reaction of serum specimens with cells from donors lacking the corresponding HL-A antigen, e. g., anti-HL-A 12 antiserum reacted with PBL with HL-A antigens 2, 3 and 7 and 2) by absorption of homologous serum with autologous or isologous PBL possessing or lacking corresponding HL-A antigens.

When applicable, possible "false-positive" reactions due to the presence of compatible blood group antigens and isohaemagglutinins, that is, e. g., in a situation where the target cell donor's PBL from a blood type "A" individual must be evaluated with serum from a blood type "B" individual possessing high titres of anti-A, may be eliminated by absorption of the test serum with blood group specific substances A and B or with appropriate RBC's, until as evaluated by isohaemagglutination all ABO (H) antibody activity has been removed.

Discussion

In the present report application of the fluorescent antibody method has been employed for the identification of HL-A antibodies on the surface of human PBL. In this regard, inclusion of the appropriate controls, maintenance of cell viability to avoid "false-positive" readings due to non-specific fluorescence obtained with non-viable cells; familiarity with the histological localization and pattern of IF staining reactions obtained on PBL with membrane-bound Ig and with antibodies other than HL-A antigens, e. g., ABO (H) antigens, are factors of particular import for the successful application of the present method.

Cognizant of the above pitfalls, it is proposed that the identification of HL-A antibodies, particularly weakly reactive or non-C' fixing, by IF might be useful in the detection of pre-existing humoral sensitiztion to donor alloantigens in prospective transplant recipients.

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