Brief Definitive Report

RECOGNITION BY PREGNANCY SERUMS OF NON-HL-A ALLOANTIGENS SELECTIVELY EXPRESSED ON B LYMPHOCYTES*

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The value of pregnancy serums for HL-A typing is well recognized (1). Most of the specific antibodies in these serums arise through immunization of the mother by the foreign HL-A antigens of the fetus. These determinants are found on all peripheral blood lymphocytes and are primarily detected in microcytotoxicity assays (1, 2).

In addition to the HL-A antigens, the histocompatability complex on the lymphocyte surface also includes the structures determining the mixed lymphocyte reaction (MLR). Although these remain undefined, certain pregnancy serums are known to selectively inhibit the MLR (3), and the serologic characterization of these MLR-related structures has been initiated (4). In the mouse an additional series of serologically defined determinants, the Ia system has been described recently that appears to map with immune response genes (5).

In the present study a large scale screening program of pregnancy serums was initiated in an effort to detect isoantibodies other than those to HL-A antigens which might relate more directly to the specificities detected in the MLR system. A modified procedure of indirect immunofluorescence was utilized for the analysis of a variety of different B and T lymphocytes exposed to the sera. A significant number of the sera showed selective staining of B cells with a clearly defined specificity pattern for cells from different individuals.

Materials and Methods

Cell Preparation. Mononuclear cells were isolated on Ficoll-Hypaque discontinuous gradients from the peripheral blood of pregnant women, nonpregnant normal individuals, and patients with chronic lymphatic leukemia. Single cell suspensions of thymus were similarly purified. The cells were washed three times with phosphate-buffered saline, 0.1% glucose (PBS-G) and suspended in Hanks' balanced salt solution. The cells were incubated with latex particles for 1 h at 37°C and washed twice with PBS-G. They were suspended in PBS with 2% bovine serum albumin and 0.02% sodium azide (PBS-BSA) at a concentration of 2×10^7 lymphocytes per ml (6). Human lymphoid lines were maintained in medium RPMI 1640 supplemented with 10% fetal calf serum. They were gently washed with PBS-BSA three times and suspended at a concentration of 1×10^7 per ml. Preparation of enriched T- and B-lymphocyte populations was made by Ficoll-Hypaque discontinuous gradient to

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separate nonrosette-forming B lymphocytes from the T cells that rosette with sheep erythrocytes as described previously (7).

Serum Preparation. Normal serums were obtained from male and female laboratory personnel that have not been isoimmunized. Unselected pregnancy serums were obtained at the time of delivery. The serums were used without dilution after heat inactivation.

Immunofluorescence. Indirect immunofluorescent staining was performed as described previously (6) with modifications. Briefly, 5×10^5 lymphocytes were incubated for 30 min with 0.025 ml of either a pregnancy serum or a normal serum in 10 x 75 mm plastic tubes precoated with BSA. After four washings with PBS-BSA, the lymphocytes were stained for 30 min with the $F(ab')_2$ fragments of a rabbit antiserum specific for human IgG (8). After three washings with PBS-BSA, the cells were processed for examination by fluorescent microscopy. All the above procedures were performed at room temperature. Direct staining with $F(ab')_2$ reagents specific for μ- and δ-determinants was used to detect lymphocytes with surface immunoglobulins as described previously (8). The presence of an Fc receptor was detected by incubating the cells at 4°C with 10 μg of human IgG labeled with tetramethyl rhodamine and 0.05 ml of whole rabbit anti-IgG conjugated with tetramethyl rhodamine, added consecutively. This method gave results in detecting Fc receptors on lymphocytes similar to the aggregated IgG assay used previously (7).

Cytotoxicity. Screening of serums was carried out on a panel of normal lymphocytes, and all cell typing was performed by the one state microcytotoxicity method according to Kissmeyer-Nielsen (2). Subsequent microcytotoxicity, using the selected serums, were performed by the two-stage assay (9).

Results

Since preliminary results indicated that antibodies specific for B cells were of special interest, 320 pregnancy serums were screened by immunofluorescence for IgG antibodies that reacted with either of two B-cell lines, RPMI 1788 and B35M. Only strongly staining serums as shown in Fig. 1, were selected. Serums with antibodies cytotoxic for a panel of HL-A-defined peripheral blood lymphocytes (PBL) were excluded leaving 32 that showed strong staining of one or both B-cell lines. These serums were then tested against normal PBL by immunofluorescence. Many brightly stained from 5 to 20% of the PBL including the B cells and did not detect determinants present on the vast majority of T cells as shown in Table I. Several serums stained T cells weakly and required absorption to eliminate noncytotoxic anti-HL-A antibodies. In Table I the number of cells

TABLE I

Percent Staining of Peripheral Blood Lymphocytes from Representative Individuals by Pregnancy Serums in Indirect Immunofluorescence

Serum	Cells from normal individuals			Cells from pregnancy serum donor	
	CD	FJ	MH	127	057
Pregnancy serum					
058	15	<2	18	2	12
057	10	<2	13	10	<2
127	5	<2	11	2	NT
111	12	8	2	10	NT
Normal serum	<2	<2	<2	<2	<2
Surface markers					
Membrane Ig*	8	3	11	8	11
Fc receptor	15	8	14	6	10

* Membrane Ig refers to the % of cells bearing either IgM or IgD or both. NT, not tested.

stained equalled or slightly exceeded the number with Fc receptors and was greater than the number with membrane Ig. These serums gave patterns of considerable specificity in their reactions with different normal subjects as shown in Table I, and did not react with autologous lymphocytes. The background staining of normal serums was under two percent.

Monocytes from different subjects also were brilliantly stained and this staining was found in association with positive B-lymphocyte staining as shown in Fig. 1; it could be readily differentiated from the background staining seen upon incubation with normal serums. Thymocytes from three individuals were studied. At least 90% of the cells were negative but 10% of the cells stained in one instance. Some staining of stimulated PBL T cells was also observed. The staining reactions of purified preparations of normal B and T lymphocytes from PBL are shown in Fig. 1.

The pattern of specificity shown by the serums for determinants on peripheral blood lymphocytes was further explored on B-cell lymphoid lines and leukemic B lymphocytes (Table II). The positive staining of the B-lymphoid lines was often extremely brilliant, and included 100% of the cells; it contrasted with the negative reactions which for most lines was the absence of any staining of viable cells. Certain lines showed weak positive fluorescence, with all normal serums; if this caused interpretive difficulties, the lines were excluded from the study. All serums failed to stain the T-cell line MOLT 4F. The staining of the leukemic B lymphocytes from nine patients showed a similar pattern of positive and negative reactivity. Surface Ig, intracellular Ig, Fc receptors, and HL-A type were determined and did not appear to influence the results of the staining with the B-cell lines or leukemic lymphocytes.

The antibody specificity was also readily demonstrated in a two-stage microcytotoxicity assay. The results of fluorescence and cytotoxicity were in general agreement. For example, serum 172 shows no cytotoxicity for cell lines 6410 and MOLT 4F, 72% killing of 7301 and 29% for 1788; and serum 007 kills 100% of cell lines 6410 and B35M, 68% of 7301, and 35% of 1788 and is without cytotoxicity for MOLT 4F. Similar results were obtained with enriched preparations of peripheral blood B cells.

Discussion

The studies described above demonstrate that some pregnancy sera contain antibodies that are reactive with B cells and not visibly with T lymphocytes. B-cell lymphoid lines, normal peripheral blood B cells, and CLL B cells all showed positive fluorescence reactions while preparations of normal peripheral blood T cells and thymocytes were essentially negative. These sera were selected in a large scale fluorescence screening procedure utilizing the B-cell lymphoid lines and normal peripheral blood T cells as target cells. HL-A antibodies were excluded by their reactivity with both types of cells by fluorescence as well as through the usual cytotoxic screening with a panel of cells. However, it was clear that some sera containing HL-A antibodies also contained the B-cell reactive type and after absorption either with T cells or platelets were specific for B cells.

Through the use of a panel of 11 different B-cell lymphoid lines, clear differential patterns of fluorescence were obtained with individual selected sera.

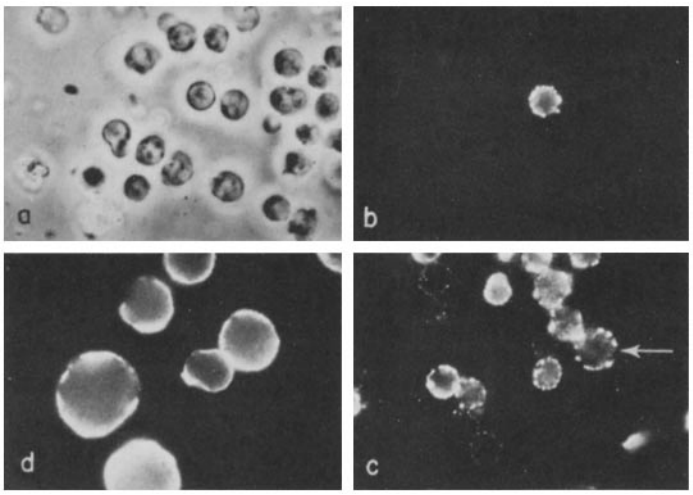

FIG. 1. Illustration of the fluorescence obtained on incubating the selectively screened pregnancy serums with purified preparations of normal peripheral blood cells from a positive donor and with cells from a positive lymphoid line. (*a*) phase micrograph of an enriched preparation of rosette-forming T lymphoctyes. (*b*) fluorescent micrograph of the same field showing only a single-stained lymphocyte. (*c*) fluorescence of a comparably enriched non rosette forming preparation of mononuclear cells composed primarily of B lymphocytes. The arrow indicates a monocyte identified under phase microscopy by latex ingestion. (*d*) bright fluorescence of one positive B-cell lymphoid line.

Cells positive with one serum were negative with another and vice versa. It was apparent that a large number of different antigen-antibody systems were involved and considerable further work will be required to delineate the specific markers. Analyses of peripheral blood B cells from individual donors proved more difficult but through a series of improvements in the indirect fluorescence method, particularly the use of $F(ab')_2$ reagents, these problems were largely overcome. Again differential patterns of normal B cells similar to those for the cell lines were obtained. Because of the low numbers of B cells in normal peripheral blood, it frequently proved advantageous to concentrate the B-cell population. Monocytes were also positive in individuals where the B cells were positive and negative in the others. This was also true of the so-called "third population" of lymphocytes with Fc receptors but no surface IgM and IgD (8). The maternal cells of the serum donor were clearly negative with autologous serum in each instance studied. Since it was apparent from these data as well as family studies that another alloantigen-antibody system was involved, it was termed HL-B. However the possibility of multiple genetic systems remains.

Assays by cytotoxicity paralleled those by fluorescence. Previous failures to detect these antibodies by cytotoxic analysis appears to be due to the low numbers of B cells in normal peripheral blood. This can be overcome by specific enrichment of the B cells. Previous studies of human lymphoid lines with HL-A antisera have led to the postulation that "blast" antigens are present and explain a variety of anomolous reactivities (10, 11). Absorption studies demonstrated that the antigens described above were not of this type. Studies in the author's

TABLE II

Representative Reactions Given by Selected Pregnancy Serums on Lymphocytes from Leukemic Patients and Lymphoid Lines in Indirect Immunofluorescence

Pregnancy serum number	Lymphoid lines								Leukemic B lymphocytes			
	RPMI 6410	Daudi	RPMI 1788	7301	RPMI 8866	TH	B35M	MOLT 4F	A	B	C	D
058	3*	2	2	0	±	2	4	0	2	2	0	2
057	0	0	0	3	2	2	4	0	3	2	2	2
127	0	0	0	1	2	2	2	0	2	0	0	3
172	0	0	3	3	±	0	0	0	0	2	0	3
111	1	3	3	3	2	2	3	0	0	2	3	3
007	4	3	1	2	4	4	4	0	2	2	2	3
Normal	0	0	0	0	±	0	0	0	0	0	0	0

* Numbers (1–4+) refer to definite positive staining of over 90% of cells with an estimate of fluorescence intensity.

laboratory of a special serum PL which had been shown to block strongly the MLR reaction (3) indicated that this serum reacted with B cells in a manner similar to the sera under discussion (4). Recently the technique of antibody-mediated lymphocyte cytotoxicity has provided evidence for alloantibodies relating to MLR determinants (12).

It appears likely that the HL-B antigens described in this report resemble the Ia antigens in the mouse. These map with immune response genes and specifically those of the MLR system (13). Certain of the sera used in the present study were analyzed for MLR inhibition and inhibition of specific stimulator cells was obtained. The Ia antigen in some studies has been shown to be predominantly on B cells but evidence implicating T cells also has been obtained (14).

Summary

A group of alloantibodies are found in pregnancy sera which react with antigens present on B lymphocytes and monocytes but are not detectable on the vast majority of unstimulated T cells. This specificity distinguishes them from HL-A antibodies which react with both cell types. They were readily recognized through indirect fluorescent antibody analysis by employing the combination of B-cell lymphoid lines and normal peripheral blood T cells. Different sera gave a variety of patterns of reactivity with a panel of 11 lymphoid lines. Similar differential patterns were also observed with normal B cells from different individuals particularly after concentrating the B cells. The antibodies were also cytotoxic to B cells and this procedure gave parallel results to the fluorescence method.

The pattern of reactions obtained indicated a very heterogeneous system similar to that for HL-A. Special study of certain of the sera provided evidence that the lymphocyte-defined determinants of the mixed lymphocyte reaction system were involved. For convenience the term HL-B has been employed for these antigens.

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