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Isolation of Histocompatibility Antigens and of Several B Cell Specific Proteins from Cultured Human Lymphocytes

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The first part of this paper will describe the purification of HL-A antigens from human lymphoblast lines (B cells transformed by Epstein-Barr virus [EBV]). The second part will deal with several other polypeptides from human lymphocyte membranes which were first detected as impurities in the HL-A antigen preparations. The latter are extremely interesting and some of them appear to be B cell specific determinants of the human lymphocyte.

HL-A Antigens from Human Lymphocytes

A number of attempts had been made to purify HL-A antigens from human lymphocytes, notably by Sanderson and Batchelor (1) who used splenic lymphocytes. Very small amounts were obtained from this source. Cultured lymphoblasts appeared to be a preferable source, both because they were available in potentially larger amounts than human spleens and because an experiment with a single human spleen cannot be repeated. However, cultured lymphoblasts are available in reproducible supply and do not seem to have any altered HL-A specificities. Remarkably, these lymphoblasts also contained far more HL-A antigens than splenic or peripheral blood lymphocytes. This was shown by absorption experiments with HL-A antisera using three types of cells, all from the same individual (RH)(Table 1) (2). Peripheral blood lymphocytes from RH had a

TABLE I
Absorption of Anti-HL-A Alloantisera with PBL, PHAL, and RH-1 Cells

		Absorption Endpoints (cells/well x 10^{-4})			Ratio of Absorption Endpoints	
Specificity	Serum	PBL	PHAL	RH-1	PBL/PHAL	PBL/RH-1
HL-A3	BC	34	2.6	0.9	13	38
W-28	MWS	14	1.5	0.8	9	18
HL-A7	SAN	15	1.2	0.4	12	38
HL-A27	DAL	21	1.8	0.4	11	52
			Averages		11	36

very low absorptive capacity for these antisera. PHA-stimulated lymphocytes had a greatly enhanced capacity and RH lymphocytes transformed by EBV and growing continuously in cultures had an enormously enhanced absorptive capacity for HL-A antisera, i.e. the representation of HL-A antigens on the surface of the cultured lymphocyte transformed by EBV was in the range of 20-50 fold greater than peripheral blood lymphocytes;

all four of the HL-A specificities of RH cells were similarly affected. This enhanced representation was specific in that other membrane markers, such as 5'-nucleotidase or radioiodinatable surface protein, were increased in the transformed cell only 2-3 fold, the same as the increase in surface area. One interpretation is that the virus itself induced or enhanced expression of the antigen in some way. Alternatively, the virus may have selected for transformation a subpopulation of B lymphocytes which already had an enhanced representation. The explanation is not known but the fact that the HL-A antigens are so much more densely represented on the cultured lymphoblasts has made possible their isolation in relatively large amounts.

a. Preparation of HL-A antigens after papain solubilization. Two principle methods have been used for solubilization of HL-A antigens. They are: 1. treatment with papain, and 2. solubilization with detergent. Preparation of HL-A antigens from the cell line RPMI 4265 after papain solubilization (3) is shown in Table II. The procedure for isolation

TABLE II
Purification of Papain-Solubilized HL-A Antigens from 4265 Cells

Purification Step	Protein (mg)	% Recovery of Inhibitory Units		Specific Activity (Inhibitory Units per mg)		Purification
		HL-A2	HL-A7	HL-A2	HL-A7	
1. Cell Membrane	225	100%	100%	3,150	320	(1)
2. Papain Digest	45	32	41	5,000	660	1.5-2
3. CM-52 Chromatography	-	22	28	-	-	-
4. Sephadex G-150 Chromatography	6	22	28	25,800	3,330	8-10
5. DE-52 Chromatography	0.5 (HL-A2 0.5 (HL-A7,12)	16	14	253,000	20,000	60-80

is not difficult, requiring only four steps. The most interesting feature is that only about 70-fold purification was needed to obtain pure HL-A antigen from cell membranes, i.e. something in the order of 1-2% of the total membrane protein in the cultured lymphoblast is HL-A antigen. That is a very large representation of a single protein on the lymphocyte membrane surface. The yield of HL-A antigens was about 4 mg per 50 gm of cells, i.e. about 80 mg/kg. The cells used were homozymous for HL-A2 at the first histocompatibility locus and had HL-A7 and 12 at the second. HL-A2 has a charge difference which distinguishes it from most of the other specificities. It is readily separated on DEAE-cellulose chromatography from a mixture of HL-A7 and HL-A12 as the last step of purification. Separating the allelic specificities from each other is one of the biggest problems in this field. It is relatively easy to obtain pure HL-A antigens, i.e. a mixture of the four specificities. Of course, the most interesting part of the chemistry requires that they be separated. Starting with doubly homozygous cell lines would greatly reduce the problem.

Our first interesting finding was that these antigens contain two subunits (4,5): a heavy chain which is glycoprotein and a light chain which is now known to be β_2-microglobulin (a protein first isolated

from human urine). SDS gels of the HL-A2 antigen preparation showed the heavy chain to have a molecular weight of 34,000 and the light chain to have a molecular weight of 12,000 (β_2-microglobulin). SDS gels of the HL-A7,12 mixture showed a doublet of 37,000 molecular weight in addition to β_2-microglobulin. One of the doublet glycoproteins may be HL-A7 and the other HL-A12. However, since these gels are denaturing gels, the glycoproteins could not be recovered to prove that point.

Despite the apparent purity of the HL-A antigen preparations, isoelectric focusing revealed considerable heterogeneity (3). For example, in the HL-A2 preparation at least four bands with HL-A2 antigenic activity were seen. The most interesting possible interpretation of this heterogeneity was that there was heterogeneity in the amino acid sequence and, therefore, that some kind of V region might exist in the HL-A antigens. However, the heterogeneity turned out to be due to variability in the number of sialic acid residues on the molecule (6). The HL-A2 preparation was treated with neuraminidase as a function of time. The initial preparation contained a species with two sialic acid residues as the major component but there were also species with three and species with one sialic acid residue. As the result of treatment, a preparation which was sialic acid free was obtained and all the heterogeneity disappeared. The same result was obtained for the HL-A7,12 preparation.

Are these antigen preparations really pure? To approach that question, the HL-A antigen preparations were labelled by reductive methylation with formaldehyde and sodium borohydride (7). About two methyl groups/mole were introduced on the ε-amino groups of lysine of the molecule. This treatment did not alter immunological activity at all. Essentially 100% of these labelled preparations of HL-A2 antigens formed a specific complex with HL-A2 antisera. No significant complexation was observed with normal serum or with specificity controls (HL-A antisera with specificities other than HL-A2).

However, when the HL-A7,12 preparation or another HL-A antigen preparation containing HL-A3, W25, 12 and 27 were used, only about 70% of the total antigen could be complexed (7). With the HL-A7,12 preparation, about 40% complexation was obtained with HL-A7 antiserum, 30% with HL-A12 antiserum and a total of 70% with a mixture of antisera. What does that mean? The residual 30% of material could be third locus or other unidentified HL-A antigens, denatured antigens, or some other unrelated material co-purifying with HL-A.

Another evidence of purity is the single common amino terminal group found in the HL-A antigen preparations (7). The three preparations available all had glycine as the N-terminal amino acid of the heavy chain. An isoleucine residue was also found; it is the N-terminal residue of β_2-microglobulin.

b. Preparation of HL-A antigens after detergent solubilization. Most membrane proteins are solubilized by detergents which have HLB (hydrophilic lipophilic balance) numbers in the range of 12-14. The HLB number is an empirical measure of a detergent's tendency to make oil-in-water or water-in-oil emulsions. The solubilization of HL-A antigens appeared similar to the solubilization of bacterial membrane proteins except for the fact that a group of relatively hydrophilic Brij detergents appeared to be relatively selective in solubilizing HL-A antigens (8).

The purification of detergent-soluble material using an anti-β_2-microglobulin immunoabsorbent column is summarized in Table III. An earlier procedure (8) yielded partially purified material. After membrane

TABLE III

Purification of Detergent Solubilized HL-A from 150g of J. Yoder Cells

Purification Step	mg	HL-A2 Inhibitory units	HL-A7 Inhibitory units	% Recovery		Specific Activity (inhibitory units/mg)		Fold Purification
				HL-A2	HL-A7	HL-A2	HL-A7	
Detergent-solubilized Membrane	750	248,000	102,000	100	100	330	140	(1)
Lectin Column Chromatography	111	210,000	85,000	85	83	1890	760	5.6x
Anti-β_2-microglobulin Column	-	157,000	60,000	63	59	-	-	
Bio-Gel A-5m Column	7	133,000	50,900	54	50	19000	7300	55x

preparation and solubilization in detergent, the next steps are passage through a lectin affinity column, absorption on an anti β_2-microglobulin affinity column and subsequent elution with purified soluble β_2-microglobulin, and removal of the excess β_2-microglobulin on an Bio-Gel A-5m column. The purification required to get pure antigen was only about 50-fold over the detergent-solubilized membranes and the yields were on the order of 50%. About 7 mg of HL-A antigen were prepared from 150 g of cells. The anti-β_2-microglobulin column has also been used successfully on a small scale without the lectin column step(R. Robb, unpublished). Alternatively, repeated agarose gel filtration after passage through the lectin column also yielded pure antigen and may be more applicable to large scale work (T. Springer, unpublished). The detergent solubilized HL-A antigens also contained two polypeptides, a heavy chain of 44,000 daltons, and a light chain of 12,000 daltons, thus showing that the previously observed structure was not the result of proteolysis by papain.

Treatment with papain showed that there is an intermediate in the degradation of the heavy chain (MW 44,000) which has MW 39,000 (8). The cleavage by papain proceeds in two steps removing a maximum of 5,000 daltons at each step to yield finally the 34,000 MW heavy chain of the papain solubilized HL-A antigens. β_2-microglobulin is resistant to papain under these conditions.

c. Homology between HL-A antigens and immunoglobulins.

A number of laboratories including our own have demonstrated that the light chain of HL-A is identical to β_2-microglobulin (5,9,10,11). Moreover, β_2-microglobulin was sequenced and was shown to have sequence homology to the immunoglobulin domains, especially to the C_3H domain of IgG (12). Several other points of homology between the HL-A antigens and the immunoglobulins included the two-chain structure and limited proteolysis by papain. It seemed logical therefore to look further. Did the HL-A antigens have a four-chain structure as does IgG, for

example, i.e. two heavy chains and two light chains? Reducing agents had always been used in purification. Preparations were then made of the detergent solubilized HL-A antigens without reducing agents present (13). On agarose gel filtration the peak of HL-A activity was considerably broader and tailed towards the high molecular weight end. The column effluent was divided into four pools, each of which was subjected to radioiodination and double-antibody precipitation. Pool 4, the lowest molecular weight pool, contained a 44,000 molecular polypeptide, plus the 12,000 molecular weight polypeptide but little material of higher molecular weight; on reduction with mercaptoethanol exactly the same pattern was obtained. However, using the highest molecular weight pool, there was no HL-A antigen at 44,000 daltons, a small amount of material at about 85,000 daltons and a very large amount of material with considerably higher molecular weight. When this material was reduced, a 44,000 daltons polypeptide was obtained plus the 12,000 daltons polypeptide.

Another way of looking at these pools is by two-dimensional SDS gel electrophoresis using no reducing agent in the first dimension and adding mercaptoethanol in the second dimension. In the low molecular weight pool only the 44,000 and 12,000 daltons polypeptides were seen (in addition to impurities of 29,000 and 34,000 daltons). All of these polypeptides were on a diagonal line. Any protein which is disulfide-linked (and is therefore reduced in the second dimension of the gel) will lie below this diagonal.

In the higher molecular weight pool from the agarose column the 44,000 daltons polypeptide is reduced in amount. Another polypeptide appeared below the diagonal at about 85,000 daltons in the first dimension but at 44,000 daltons in the second. In the highest molecular weight pool almost no monomer at 44,000 daltons was present and virtually everything was an oligomer which was excluded from the gel in the first dimension. In the second dimension it was reduced and had a molecular weight of 44,000. It seems clear that the HL-A antigen preparation contained a heavy chain in the oligomeric form. Observations which led to the same conclusion had also been made by two other laboratories. What is the significance of these observations? Is the polymerization an artifact which occurs after isolation of the HL-A antigens -- i.e. did heavy chains in the preparation become crosslinked to each other during isolation? Two kinds of experiments to examine that possibility have been carried out. If, indeed, the antigens are present in a tetrameric structure in the membrane before solubilization, then a chemical crosslinking reagent should crosslink the chains in various ways forming at least heavy chain dimers and light-heavy dimers. The only product obtained with crosslinking reagents was a dimer containing a light chain and a heavy chain. A dimer containing two heavy chains was not formed. In another set of experiments cells were treated with iodocetamide to block all the free SH groups before isolating the antigens. Under these conditions little or no oligomer was present in the isolated HL-A antigens. Both of these experiments seem to suggest that HL-A antigens do not exist in the membrane as oligomeric forms.

Despite this there is other evidence of homology to immunoglobulins(13). The heavy chain of 34,000 consists of a carbohydrate of about 10,000 daltons and a polypeptide of about 24,000 daltons, i.e. the polypeptide is two "immunoglobulin domains" in size. If it is homologous to immunoglobulin, it should contain two intrachain S-S bridges. In all three preparations of solubilized antigens there were four half cystines involved in intrachain bridges, i.e. two bridges for each 24,000 daltons or one intrachain bridge for each "immunoglobulin domain". That result is consistent with an homology of the heavy chain of HL-A antigens to immunoglobulins. It remains to be shown that in fact the intrachain bridges are distributed one in each 12,000 daltons of polypeptide in

the heavy chain.

In the detergent solubilized molecule, there are two additional easily reduced SH groups, not present in the papain solubilized molecule. These additional SHs must be located in the hydrophobic region of the molecule which is presumably buried in the membrane. They provide the potential for intrachain bridges leading to the formation of oligomers, but, as indicated above, presently it is uncertain whether or not those oligomers have any biological significance.

B Cell Specific Antigens from Human Lymphocytes

At an early stage of purification the HL-A antigen preparations (obtained after papain solubilization) all contain impurities in varying amounts with molecular weights of 70,000, 30,000, 23,000 and 13,500. The latter is distinguishable from β_2-microglobulin.

These materials were separated by careful gel filtration. In addition to the peak of HL-A antigen, three additional peaks of protein were obtained which calibrated on the gel column at molecular weights of 75,000, 135,000 and in the excluded volume. The 75,000 molecular weight material was composed of two polypeptides with molecular weights of 23,000 and 30,000; they may be present in the ratio 2:1. The material which calibrated at a molecular weight of 135,000 on the Sephadex column was composed of apparently identical polypeptides of molecular weight 70,000 each. The material of very high molecular weight in the excluded volume of the column contained a single polypeptide of molecular weight 13,500, apparently highly aggregated. The purity of some of these preparations is illustrated by SDS gels (Figure 1) (R. Humphreys, unpublished).

Rabbits were immunized with all of these preparations. The properties of the antisera which were obtained are extremely interesting.

a. Rabbit anti-p23,30 serum

1. Lysis of peripheral blood lymphocytes and lymphoblast lines. Only a fraction of peripheral blood lymphocytes were lysed by the antiserum in complement-mediated cytotoxicity assays (Figure 2). However, two B cell lymphocyte lines, RH-1 and IM-1, were totally lysed at antiserum dilutions of 1:2000. When peripheral blood lymphocytes were separated into B, T and null cells, the T cells were not lysed at all; the B cells were completely lysed and a fraction of the null cells were lysed. In separate experiments with the null cell population the population lysed by anti-p23,30 serum was found to bear the EAC rosette receptor and to cause the ADCC reaction. The null cell population which was not lysed by the anti-p23,30 serum did not bear the EAC rosette receptor and did not participate in the ADCC reaction. A anti-p23,30 serum blocked the ADCC reaction (but not MLC reactions). By contrast, anti-β_2-microglobulin serum lysed all of these populations of cells.

Peripheral blood lymphocytes of 40 individuals were separated into T cells and B cells. All of these individuals' B cells were lysed by the sera and, at the low dilution used (1:10), some of the T cells are also lysed. However, at 1:500 or 1:1000 the antiserum was absolutely specific for B cells of the separated populations. T cell lines and B cell lines established from the same individuals were also examined. Three such pairs of lines were available. Again, in each ease only the B cell line was lysed at high dilution (1:500). At low dilution some partial lysis of one of the T cell lines was observed.

2. Separation of cells in the fluorescence activated cell sorter. In the Herzenberg fluorescence activated cell sorter the difference between T, B and null cells was dramatically observed. Cells were treated with anti-p23,30 serum and then with FITC conjugated goat anti-rabbit IgG. The fluorescence activated cell sorter yields data regarding both the number of cells and their relative fluorescence. A very high fluorescence was obtained with B cells, but no reaction was observed with T cells. A population of null cells was shown to react with the p23,30 antiserum (Figure 3).

3. Precipitation of polypeptides from [^{35}S]methionine internally labelled and detergent solubilized membranes. Another way of examining the specificity of these antisera is to radiolabel membranes and ask what polypeptides are precipitated. This question was of special interest with the p23,30 antiserum because it had been obtained by immunization with p23,30 polypeptides prepared after papain solubilization. The native form of the p23,30 complex might be different in the membrane. When radiolabeled membranes were solubilized in detergent and their extract treated with p23,30 antiserum, three polypeptides were observed in the precipitate with molecular weights of 39,000, 34,000 and 29,000. Apparently the p23,30 polypeptides originated from these. The precursor product relationship among these has not yet been elucidated.

Many HL-A antisera contain additional antibodies specific for polypeptides other than HL-A (obtained by double antibody precipitation from radiolabeled membranes). DAL (an HL-A27 antiserum), MWS (a W28 antiserum) and BC (an HL-A3 antiserum) all brought down a small amount of the 30,000 daltons polypeptide. BEL (an HL-A27 antiserum) is very interesting because it contains an antibody directed against a 70,000 daltons component as well as an antibody directed against the 30,000 daltons component.

Using a rabbit anti-β_2-microglobulin serum only the 44,000 daltons heavy chain of the HL-A antigens and the 12,000 daltons β_2-microglobulin were precipitated. No polypeptides corresponding to p39,34,29 were observed. At least as defined by this type of experiment, no polypeptide other than that of 44,000 daltons is associated with β_2-microglobulin in the human lymphocyte membrane.

4. Lysis of various Amish cell lines by Amish antisers and its inhibition by p23,30 antigens. The p23,30 antigen has been obtained from three different cell lines: IM1 (presently available as the purest of the preparations), RPMI 4265 and JY. JY is a member of the Indiana Amish community, a highly inbred human population. A number of cytolytic anti-sera from the multiparous women of this community to do contain HL-A antibodies but apparently have antibodies directed against other lymphocyte membrane components (14). These sera were used in cytotoxicity assays against four cell lines, also derived from the Amish population (Table 4, see next page). The antisera used lysed all four of the cell lines. Several patterns are evident. First of all, JY p23,30 antigen inhibited lysis of the JY cell line by all of the antisera; the same was true of JY p23,30 antigen as an inhibitor of lysis of cells of his relative, PY. An interesting pair of antisera are antisera 35 and 76. Antiserum 35 which lysed all four cell lines was blocked by the IM1 p23,30 antigen in each case. Antiserum 76, however, was not inhibited by the IM1 p23,30 antigen; by contrast, it was blocked by the p23,30 antigen from RPMI 4265 cells in every case. All of the data suggest that the p23,30 antigens from IM1, 4265 and JY cells are alloantigens; some of them inhibit some of the Amish alloantisera and others inhibit other Amish alloantisera. Antiserum RMB is interesting because it was inhibited by all three of the p23,30 antigens.

TABLE IV
Inhibition by p23,30 of Cytolyis of
Amish Cell Lines by Amish Antisera

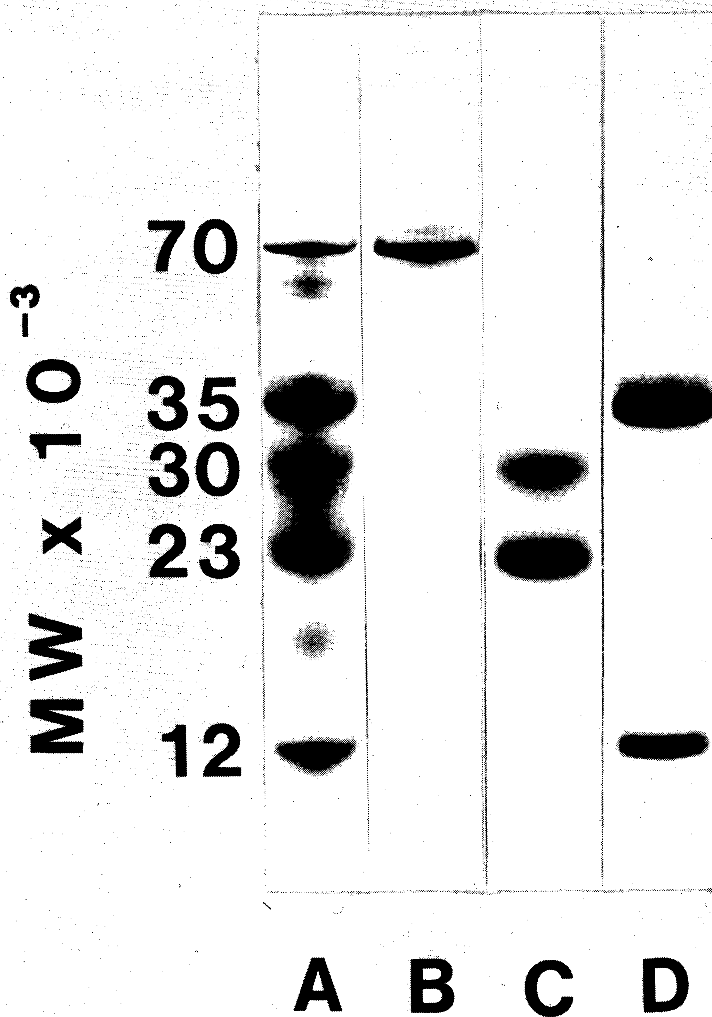
Amish Cell Line	Cell Source of p23,30 added	Amish Antisera 35	76	192	289	590	RMB
KL	IM-1	+	-	-	+	-	+
	RPMI 4265	-	+	-	-	-	+
	JY	+	+	+	+	-	+
SL	IM-1	+	-	-	-	-	+
	RPMI 4265	-	+	-	-	-	+
	JY	+	+	+	+	-	+
PY	IM-1	+	-	-	+	-	+
	RPMI 4265	+	+	-	-	-	+
	JY	+	+	+	+	+	+
JY	IM-1	+	-	-	+	-	+
	RPMI 4265	-	+	-	-	-	+
	JY	+	+	+	+	+	+

Possibly it recognizes a determinant common to all of them.

5. Use of p23,30 rabbit antisera in purification of p23,30 antigens. An interesting use of this antiserum is in following the p23,30 complex in a crude mixture, e.g. the separation of solubilized membrane proteins on a gel filtration column. The p23,30 complex, detected by inhibition of cytolysis, immediately preceded the HL-A antigens.

b. Rabbit anti-p70 serum and anti-p13.5 serum. Only a fraction of peripheral blood lymphocytes were lysed by anti-p70 serum but the RH-1 B cell lymphoblast line was lysed totally (Figure 4). B cells in separated populations of peripheral blood lymphocytes were lysed completely at a titer of 1:125. Null cells and T cells were lysed only at lower dilutions of antiserum. Similar data were obtained for rabbit anti-p13.5 serum (Figure 5). These preliminary experiments need to be extended but the data presently available suggest that several of the different antigens in the cultured human lymphoblast may be B cell specific. Like the HL-A antigens, one or more of these may be representatives of a genetic polymorphism. Certainly there is evidence that this is the case for the p23,30 proteins.

Figure 1. SDS-polyacrylamide gels of lymphocyte membrane proteins. A: HL-A antigen prepared with DTT-preactivated papain containing "contaminants" of 70,000, 30,000, and 23,000 daltons. B: Purified 70,000 daltons component. C: Purified complex of 23,000 and 30,000 daltons components. D: Purified HL-A antigen containing 35,000 and 12,000 daltons components.



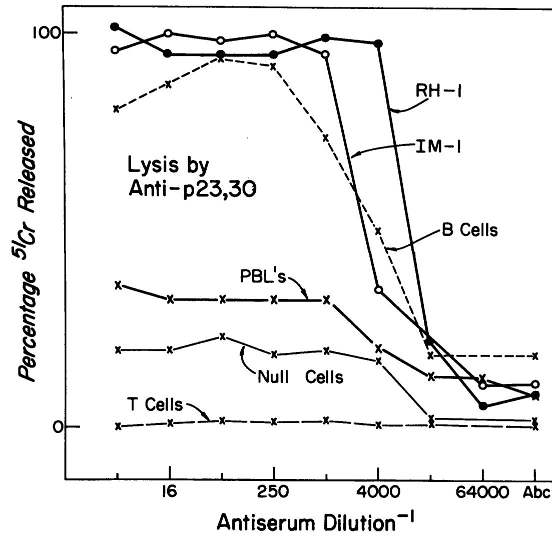


Figure 2. Lysis of several purified populations of lymphocytes by anti-p23,30 rabbit sera. The IM-1 cell line, from which the antigen used in immunization was derived is lysed completely as is the B lymphoblast line RH-1. Peripheral blood lymphocytes (PBL's) from one individual (RH) were separated into B, T and Null lymphocyte populations. The PBL's were lysed to 30% of the total population. B lymphocytes were lysed completely while T lymphocytes were not lysed at all. About 20% of Null lymphocytes were lysed.

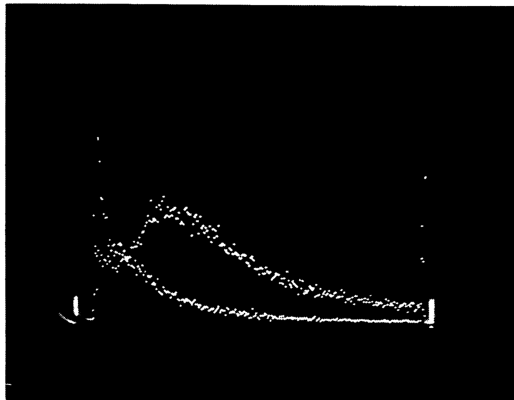


Figure 3. Fluorescence-activated cell sorter anaysis of human T and B cells reacted with fluorescinated anti-p23,30 serum. The abscissa represents relative degree of fluorescence. The ordinate represents the number of cells per fluorescent channel. The upper curve (reactive cells) represents B cells; the lower curve (non-reactive cells) represents T cells.

Figure 4. Lysis of various lymphocytes with anti-p70 serum. The cells used are described in Figure 1. Ordinate is percentage ^{51}Cr released.

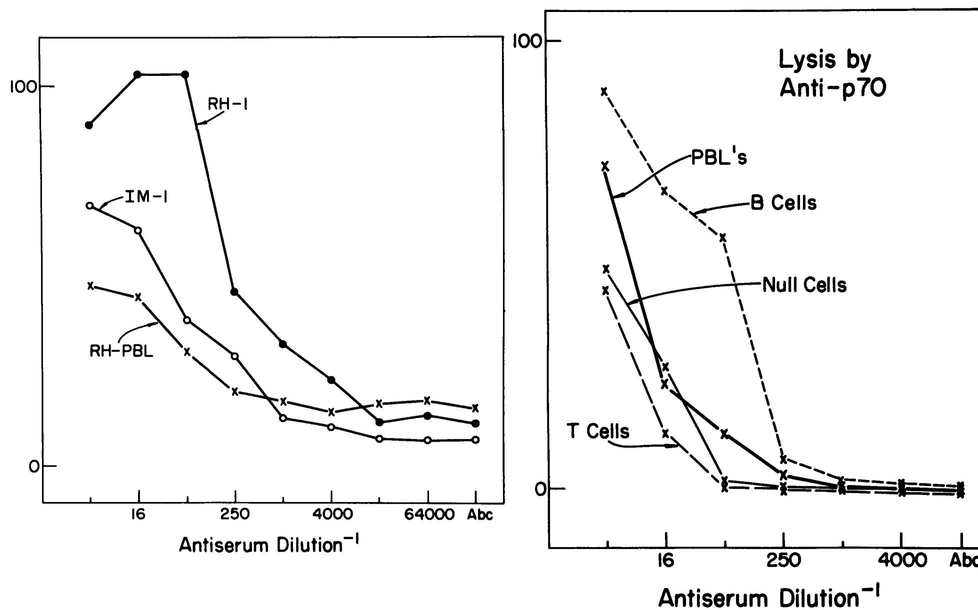
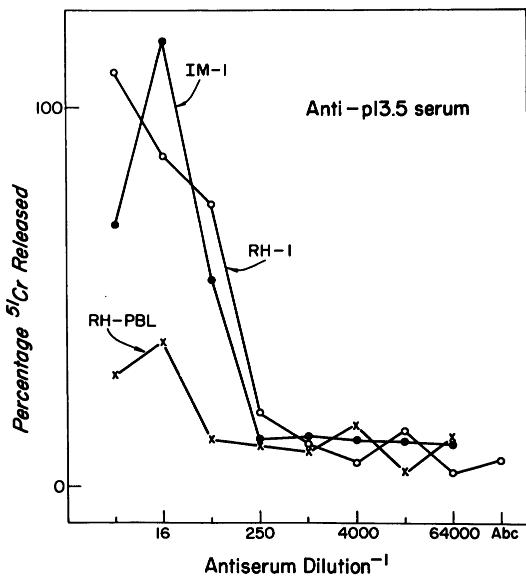


Figure 5. Lysis of lymphoblast lines and peripheral blood lymphocytes by anti-p13.5 serum. A fraction of PBL's is lysed. In separate experiments the sole reactivity of anti-p13.5 against subpopulations of PBL's was recorded against B lymphocytes.



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(Only a limited number of references have been provided, mainly those from the authors' laboratories. Complete documentation can be found in various articles in Transplantation Review, Vol. 21, 1975 and in the articles cited above.)

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