CLINICAL HISTOCOMPATIBILITY TESTING

VOLUME 2

CHARLES B. CARPENTER

A Transplantation Proceedings Reprint
December 1977

CLINICAL HISTOCOMPATIBILITY TESTING VOLUME 2

Edited by

CHARLES B. CARPENTER, M.D.

Associate Professor of Medicine
Harvard University
Director of Immunology Laboratory
Peter Bent Brigham Hospital, Boston

GRUNE & STRATTON

A Subsidiary of Harcourt Brace Jovanovich, Publishers
New York San Francisco London

INTRODUCTION

DATA regarding the genetics, structure, and function of the products of the major histocompatibility complex (MHC) are accumulating at a rapid rate. The material that follows, presented at the third annual meeting of the American Association for Clinical Histocompatibility Testing, attests to the intensity and diversity of work being carried out in man. Papers of both the invited speakers and those selected from submitted abstracts are grouped topically as far as possible. Major emphasis is now being given to definition of Ia-like B-cell antigens in man, and several aspects of technique, genetics, and role in transplantation are presented. With regard to the HLA-D locus, new evidence is presented (pro and con) regarding the specificity of in vitro primed lymphocytes to HLA-D when they are restimulated. Another product of in vitro priming, the cytotoxic lymphocyte, is also shown to have specificity to the original stimulating antigens, but as with the PLT cell, immunity to additional non-HLA-A,B,C, or D antigens is inferred because of unexplained reactions on panels of target cells.

The clinical role of the HLA system in renal transplantation and platelet transfusion, as well as new approaches to monitoring the immune responses in transplant recipients, are presented, and there are significant new data on the influence of HLA in disease processes, as well as reviews of the possible underlying mechanisms relevant to disease susceptibility and MHC gene products.

Finally, this issue ends with a symposium on granulocyte antigens. Though not HLA-linked, there is evidence for both organ-specific and allospecific antigens on granulocytes, and they may play a role in diseases with neutropenia and in transfusion therapy. They also provide a model for considering the role of non-MHC antigens in tissue transplantation.

CHARLES B. CARPENTER
Editor

CONTENTS

(Continued)

CONTENTS

(Continued)

PRIMED LYMPHOCYTE AND CELL-MEDIATED LYMPHOLYSIS TESTING

CLINICAL TRANSPLANTATION AND TRANSFUSION

(Continued)

CONTENTS

(Continued)

THE MAJOR HISTOCOMPATIBILITY COMPLEX AND DISEASE

(Continued)

CONTENTS

(Continued)

SEROLOGICALLY DEFINED ANTIGENS OF THE MAJOR HISTOCOMPATIBILITY COMPLEX

Structural Aspects of the Products of the Human Major Histocompatibility Complex

A. Fuks, J. F. Kaufman, H. T. Orr, P. Parham, R. R. Robb, C. Terhorst, and J. L. Strominger

THE STUDY of the major histocompatibility complex (MHC) and its products has led to an appreciation of the central role that this genetic region plays in various aspects of immunobiology. A large body of data attests to the understanding that has been gained of the genetic and serologic complexities of this supergene as well as its relevance to clinical transplantation.[1] In addition, more recent work, especially in the mouse, guinea pig, and rat, allows one to view the MHC in a broader biologic perspective, since it has pointed to a variety of aspects of the normal immune response in which the MHC is intimately involved. For example, antigen presentation by macrophages to T lymphocytes is regulated by the Ia specificities present on the surfaces of the interacting cells[2] and by soluble factors bearing Ia determinants.[3] The collaboration between T and B lymphocytes in the production of antibody is also restricted by the I-region specificities of the cooperating cells.[4] Furthermore, antigen-specific soluble factors that may mediate intercellular interactions (both collaborative and suppressive) in the immune response bear Ia determinants.[5,6] It has been demonstrated in a number of systems that cytotoxic T lymphocytes recognize the serologically defined histocompatibility antigens during killing directed against viral,[7] minor histocompatibility,[8] or chemically modified cell surface antigens.[9] The MHC also includes the immune response (Ir) genes, which determine responsiveness to a variety of defined antigens.[10]

In the human, the MHC products appear to be involved in stimulation in the mixed lymphocyte reaction, as well as providing the targets for killing in the cell-mediated lympholysis assay.[1] These two phenomena are likely candidates as the in vitro analogs of graft rejection. Finally, also in humans, various MHC specificities (both serologically defined and lymphocyte defined) show statistical associations with a variety of human disease states.[11] The overall picture that is emerging is of the paramount importance of the MHC in all phases of both the normal and pathologic immune response. Indeed, if the immune system is a series of networks, their interconnecting regulatory and recognitive elements are the immunoglobulins and the products of the MHC.

A major aim of our laboratory has been the purification and physicochemical characterization of the antigens specified by the human MHC in the hope that a knowledge of their structure will shed light on the molecular underpinnings of their biologic functions. This article will deal with three aspects of these investigations: the structure of the HLA-A and B antigens, data on the human analogs of the murine Ia antigens, and some experiments demonstrating the usefulness of a heteroantiserum made against these Ia-like antigens in the in vitro investigation of lymphocyte function.

PRODUCTS OF THE HLA-A AND HLA-B LOCI

The starting materials for antigen purification are human lymphoblastoid B-cell lines derived from spontaneously arising

From the Biological Laboratories, Harvard University, Cambridge, Mass.

Supported in part by the Public Health Services, National Institutes of Health Grants AI-10736 and AI-09576. H.T.O. is a fellow of the National Institutes of Health. A.F. is a fellow of the Medical Research Council of Canada.

Reprint requests should be addressed to A. Fuks, Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge, Mass. 02138.

 ISSN: 0041-1345.

lymphoid malignancies or developed by in vitro transformation of human peripheral lymphocytes by Epstein-Barr virus. The cell lines utilized were JY, with the serologic phenotype HLA-A2,2;B7,7, and RPMI 4265, HLA-A2,2;B7,12. These are available in large quantities and also appear to have an increased representation of HLA antigens as compared with normal peripheral blood lymphocytes.[12] Membranes are prepared from these cultured cells and treated with papain to solubilize the HLA-A and B antigens. The soluble material is processed by gel filtration on Sephadex G-150, followed by ion-exchange chromatography on DEAE-cellulose. This last step allows separation of the HLA-A2 from the other antigenic specificities. This procedure yields essentially pure HLA antigen, which consists of two chains a 34,000-dalton large chain and a small chain of 11,600 daltons that is identical to β_2-microglobulin.[13]

An alternative purification method is to utilize the nonionic detergent Brij to solubilize the membranes and finally to obtain the HLA antigen by anti-β_2-microglobulin immunoaffinity chromatography.[14] This technique yields a molecular complex consisting of a 44,000-dalton large chain and the 11,600-dalton β_2-microglobulin. The large chains can be separated from β_2-microglobulin by denaturation and chromatography in guanidine hydrochloride and are thus made available for further analysis.

The chemical studies undertaken were then directed toward answering two broad questions. What is the structural basis for the serologically defined differences among HLA molecules? Are there similarities between HLA and other molecules of biologic interest?

Amino acid analyses of the 34,000 chain from A2 molecules from two different cell lines revealed them to be almost identical in composition.[15] More interestingly, a comparison of the amino acid compositions of A2 and B7 shows only limited differences between these two specificities. This similarity was also found when the N-terminal amino acid sequences of the two specificities were obtained. Only a single difference between A2 and B7 can be found in the first 25 N-terminal residues. Of interest has been the finding of a significant degree of sequence homology between the N-terminal sequences of the large subunits of human HLA and murine H-2.[16]

For further investigation into the structural nature of the HLA molecule, a series of degradative and analytic procedures were carried out. These included treatments with dilute acid, cyanogen bromide,[17] and NTCB,[18] the isolation and identification of various peptide fragments, and an analysis of the carbohydrate moiety of the large subunit of HLA antigen.[19] These studies have led to the following picture of the HLA molecule. It contains a large 44,000-dalton subunit inserted into the cytoplasmic membrane via a penultimate hydrophobic region of about 5000 daltons. The C-terminus, of about 5000 daltons, is hydrophilic and probably extends into the interior of the cell.[20] The molecule contains two intrachain disulfide bridges, each encompassing a region of 7000 9000 daltons. This is of course reminiscent of the domain structure of immunoglobulin. N-terminal to the two disulfide bridges is a segment of the molecule of 10,000 12,000 daltons that contains the carbohydrate moiety of HLA. This consists of a single glycan side-chain of about 3000 daltons,[19] N-linked to asparagine, and located approximately 90 100 residues from the N-terminus. Removal of almost all the sugar residues of this glycan chain can be achieved without loss of antigenic activity of the molecule. The β_2-microglobulin chain is noncovalently associated with the large chain, but the nature and sites of interaction of the two chains is not clear.

There is as yet no clear-cut information regarding the nature and location of the epitope on the HLA molecule that is recog-

nized by alloantisera. On the other hand, there are data regarding the second question noted above, and these concern the similarities between HLA antigens and immunoglobulin molecules. Thus, HLA appears to have an overall structure based on domain-like units in both its large and small subunits. Furthermore, both HLA and immunoglobulin consist of pairs of heavy and light chains, and the large subunits show limited cleavage by papain. Perhaps more striking are the sequence homologies that have been noted. The N-terminal sequence, the sequence contiguous to the third cysteine of HLA, and a region of the tryptic glycopeptide all demonstrate a degree of sequence homology with the variable regions of human and murine immunoglobulins.[17,19] It is probably premature at this point to comment on the precise significance of these findings. One should also point out that, thus far, the structural studies have not given any indication as to the natural biologic function of the HLA molecule.

B-CELL-ASSOCIATED ALLOANTIGENS

Another set of cell surface glycoprotein molecules obtainable from cultured lymphoblastoid cell lines are the Ia-like B-cell-specific alloantigens. As in the case of HLA-A and B antigens, these materials can be obtained by either papain or detergent treatment of cell membranes.[21,22] After papain digestion, the purification procedure consists of gel filtration on Sephadex G-150, DEAE ion-exchange chromatography, and removal of the remaining traces of the HLA-A and B antigens by anti-β_2-microglobulin affinity chromatography. These procedures yield a molecular complex of two noncovalently associated chains of 23,000 and 30,000 daltons (p23,30). The detergent purification scheme requires the solubilization of a membrane preparation with Brij, a nonionic detergent. The soluble materials are passed over a Lens culinaris lectin affinity column, and the bound materials, which can be eluted with α-methyl mannoside, are applied to a Bio-Gel A5M column. A complex of 55,000 daltons with subunits of 29,000 and 34,000 daltons (p29,34) can be recovered. Two component chains can then be obtained in pure form by preparative polyacrylamide gel electrophoresis.[23] The p29 and p34 subunits form a stable complex that is not dissociated by reducing agents or low concentrations of sodium dodecyl sulfate. All the evidence gained thus far suggests that the molecular complex consists of equimolar amounts of the two component subunits.[22]

Since the purified complexes (both p23,30 and the detergent-purified homologue, p29,34) were available, the question could be asked as to whether these molecules would be recognized by B-cell-specific human alloantisera. These sera were of two kinds. One set, obrained from Dr. B. Solheim, was the result of planned immunization in HLA-A, B, and C compatible individuals who differed at the HLA-D locus. Another group of sera were obtained from multiparous women and were rendered B-cell-specific by platelet absorptions (these sera were a generous gift of Dr. J. van Rood). These alloantisera have all been correlated with MLC typing and in several cases have been mapped to HLA-D. Using a ^{51}Cr-microcytotoxicity assay, it could then be demonstrated that the cytotoxic potential of the alloantisera against cultured lymphoblastoid B cells was inhibited by purified p23,30 and p29,34 complexes.[24] The inhibition pattern obtained was allospecific in that a given serum could only be inhibited by purified material from a cell line against which that serum was lytic. Another approach to this same question was the utilization of the alloantisera in double antibody immunoprecipitations of labeled antigens from internally labeled cultured B cells. The precipitates were examined by polyacrylamide gel electrophoresis and autoradiography. The specific B-cell sera tested precipitated chains of 34,000 and 29,000 daltons, that is, identical in size to

the purified molecular complex described previously.[25] Taken together, the inhibition and precipitation data suggest that p23,30 and p29,34 represent B-cell-specific alloantigens coded by the MHC in the HLA-D region.

What is known of the chemical nature of these B-cell-specific antigens? As noted previously, they consist of a two-chain, noncovalently associated complex. Both chains appear to be glycosylated.[23] The amino acid compositions of the two separated chains have been determined and are notable primarily in the similarity of the composition of p29 and p34. As well, limited peptide mapping that compared the tyrosine-containing tryptic peptides of p29 and p34 suggested the presence of significant homology between the two chains.[20] These data raise interesting possibilities regarding a common evolutionary precursor for the two subunits.

N-terminal sequencing of the first 10–12 residues of the two chains demonstrated a number of interesting points.[23] First, the N-terminals of p29 and p34 do not resemble each other. Second, the sequences of p29 and p34 available from the cell line 4265 are identical with the sequences of the corresponding chains derived from the cell line JY. Finally, sequencing of the papain-solubilized products from JY suggested that p34 gives rise to p30, and p29 to p23 upon papain solubilization of the cell membranes. One possible interpretation of these last data is that both p29 and p34 lose a C-terminal fragment upon papain cleavage, and by analogy with HLA, may thus have intra- or transmembranous portions.

The concept that these molecules are the human analog of murine Ia antigens has been strengthened by the finding of sequence homology between p34 and the large subunit of mouse Ia antigens of the murine I-C subregion. In addition, there are similarities of the sequence of p29 and the 25,000-dalton chain of guinea pig Ia.[23] Preliminary evidence also indicates that p23,30 does not lose its alloantigenic activity after treatment with a mixture of exo- and endoglycosidases and suggests, as is true for HLA-A and B antigens, that the alloantigenic activity resides in the protein portion of the molecule.[26]

Another set of observations regarding this B-cell glycoprotein complex revolves around the properties of a rabbit heteroantiserum raised by immunization with purified p23,30. This serum is B-cell-specific and is devoid of reactivity against peripheral blood (or cultured) T lymphocytes.[21] It reacts with a subpopulation of null cells that is responsible for antibody-dependent, cell-mediated cytotoxicity (ADCC).[27] The serum is capable of inhibiting the mixed lymphocyte reaction,[21] inhibits the differentiation of B lymphocytes in response to antigen and mitogen,[28] and abrogates the response of human peripheral blood lymphocytes to mumps and tetanus toxoid antigens.[29] This type of heteroantiserum may thus prove useful in the assessment of various aspects of lymphocyte reactivity and may aid in the delineation of the functional importance of the human Ia-like antigens in different phases of the immune response.

The anti-p23,30 serum has also been of diagnostic usefulness because of its differential reactivity with various types of leukemic cells and can aid in the subdivision of acute lymphocytic leukemias into different prognostic subgroups.[30]

In summary, the p23,30 and p29,34 complexes are analogous to mouse Ia antigens in several aspects: size and subunit structure, reactivity with B-cell-specific alloantisera, limited sequence homology, and functional properties of the heteroantiserum described above that are similar to those of murine Ia alloantisera.

Many critical questions regarding the structure of the products of the human MHC remain unanswered. What is the nature of the alloantigenic epitopes on these molecules? Do these molecules serve as

receptors, and, if so, what is the nature, if any, of their interaction with the intracellular cytoskeleton? Are the Ia-like molecules the product of the HLA-D locus? What are the evolutionary relationships between the MHC products and immunoglobulins, and do the similarities between these two classes of molecules reflect similarities in their functions? Can the availability of purified MHC gene products and specific antisera to these materials be exploited in the management of the transplant or tumor-bearing patient? Elucidation of these answers will require the synthesis of observations at many levels of investigation, including the structural, serologic, functional, and clinical levels.

REFERENCES

1. Bach FH: Ann Rev Genet 10:319, 1976
2. Shevach EM, Rosenthal AS: J Exp Med 138: 1213, 1973
3. Erb P, Feldmann M, Hogg N: Eur J Immunol 6:365, 1976
4. van Boehmer H, Spreant J: J Exp Med 144:617, 1976
5. Munro AJ, Taussig MJ: Nature (Lond) 256:617, 1976
6. Tada T, Taniguchi M: J Exp Med 144:713, 1976
7. Zinkernagel RM, Doherty PC: J Exp Med 141: 1424, 1975
8. Bevan MJ: Nature (Lond) 256:419, 1975
9. Shearer GM, Rehn TG, Schmitt-Verhulst A: Transplant Rev 29:222, 1976
10. Benacerraf B, Katz DN: Adv Cancer Res 21: 121, 1975
11. Svejgaard A, Platz P, Ryder LP, et al: Transplant Rev 22:3, 1975
12. McCune JM, Humphreys RE, Yocum RR, et al: Proc Natl Acad Sci USA 72:3206, 1975
13. Turner MJ, Cresswell P, Parham P, et al: J Biol Chem 250:4512, 1975
14. Robb R, Mann DL, Strominger JL: J Biol Chem 251:5427, 1976
15. Terhorst C, Parham P, Mann D, et al: Proc Natl Acad Sci USA 73:910, 1976
16. Ewenstein B, Freed JH, Mole LE, et al: Proc Natl Acad Sci USA 73:915, 1976
17. Terhorst C, Robb R, Jones C, et al: Proc Natl Acad Sci USA 74:4002, 1977
18. Ferguson W, Terhorst C, Strominger JL: (in preparation)
19. Parham P, Alpert BN, Orr HT, et al: J Biol Chem (submitted for publication)
20. Springer TA, Strominger JL: Transplant Proc 9 (Suppl 1): 21, 1977
21. Humphreys RE, McCune JM, Chess L, et al: J Exp Med 144:98, 1976
22. Springer TA, Kaufman JF, Siddoway LA, et al: J Biol Chem 252:6201, 1977
23. Springer TA, Kaufman JF, Terhorst C, et al: Nature 268:213, 1977
24. Kaufman J, Fuks A, Giphart M, et al: Proceedings Ir Gene Workshop, Asilomar, 1977
25. Giphart M, Kaufman JF, Fuks A, et al: Proc Natl Acad Sci USA 74:3533, 1977
26. Fuks A, Strominger JL: (in preparation)
27. Chess L, Evans R, Humphreys RE, Strominger JL, Schlossman SF: J Exp Med 144:113, 1976.
28. Friedman SM, Breard JM, Humphreys RE, et al: Proc Natl Acad Sci USA 74:711, 1977
29. Breard JM, Chess L, Fuks A, et al: Fed Proc 36:1262, 1977 (abstr)
30. Schlossman SF, Chess L, Humphreys RE, et al: Proc Natl Acad Sci USA 73:1288, 1976

An Immune Adsorption Technique for Analysis of Antigenic Determinants Reacting With HLA Antisera

J. P. Allison, M. A. Pellegrino, R. A. Reisfeld, and S. Ferrone

WE HAVE reported that soluble HLA antigens are found in serum as a complex of a single HLA chain, a β_2-microglobulin chain, and an associated boundary lipid.[1] The antigens co-isolate with serum high density lipoprotein (HDL) and can be readily purified by ultracentrifugal flotation. Xenoantisera to HDL-associated HLA-A9 antigens have been shown by cytotoxicity testing with large panels of lymphocytes to become specific for A9 after absorption with (1) cultured lymphoid cells expressing appropriate HLA-phenotype, (2) human red blood cells, or (3) insolubilized HDL of appropriate HLA phenotype.[2] We have reported serologic (i.e., lysostrip and Fab'_2 blocking experiments) as well as immunochemical (sequential immunoprecipitation and affinity chromatography) evidence that these A9 xenoantisera react with the same antigenic complex recognized by A9 alloantisera.[3] In this article, we will present evidence, based on a novel immune adsorption procedure, that A9 xenoantisera that are monospecific in the lymphocytotoxic test may react with more than one determinant on the A9 molecule.

MATERIALS AND METHODS

Antigen Preparation

HLA antigens were isolated from serum as high density lipoprotein (HDL) by polyanionic precipitation and ultracentrifugal flotation as previously described.[1] The hydrophilic, alloantigenic fragment of HLA-A9 was prepared by papain digestion and gel chromatography.[1]

Antisera

HLA alloantisera used were Gaulier (anti-HLA-A1), Stakenburg (HLA-A2) from NIAID, and Fe 91/4 (anti-HLA-A9) from Dr. G. Ferrara. The preparation of rabbit antisera to HLA-A9-HDL and human urinary β_2-microglobulin has been reported elsewhere.[1,2]

Serologic Assays

HLA alloantigenic activity was determined using the microcytotoxicity blocking assay previously described.[4] The titer of antisera was determined by the microcytotoxicity test using HLA-typed peripheral lymphocytes as targets.[4]

Immunoadsorbents

Gamma-globulins were prepared from antisera by fractional precipitation with Na_2SO_4. The purified γ-globulins were covalently coupled to Sepharose 4B by the method of Cuatrecasas.[5]

RESULTS

The immune adsorption procedure, as used in this study to compare HLA-A9 allo- and xenoantisera, is illustrated in Fig. 1. One-hundred microliter aliquots of immunoadsorbent were loaded by overnight incubation on a rotator at 4°C with 250 μl of HLA-A2-HDL or 250 μl of the alloantigenic fragment of HLA-A9. The supernatants were removed and assayed for HLA antigenic activity. The incubation was repeated three times to ensure maximal loading of antigen onto the immunoadsorbents. The loaded immunoadsorbents were then washed 6 times with Tris-buffered saline (pH 8.0), containing 0.5% NP-40, 1 mg/ml human serum albumin, and 5 m*M* EDTA to remove nonspecifically bound antigen, and finally washed 6 times with phosphate-buffered saline (PBS). The loaded washed immunoadsorbents were then divided into four equal aliquots and incubated overnight with xeno- or alloantisera, the immunoad-

From the Department of Molecular Immunology, Scripps Clinic and Research Foundation, La Jolla, Calif.

Supported in part by USPHS Grants AI-10180, AI-13154, CA-16069, and CA-16071. S. F. is the recipient of American Heart Association Established Investigatorship. This is publication no. 1306.

Reprint requests should be addressed to J. P. Allison, Department of Molecular Immunology, Scripps Clinic and Research Foundation, La Jolla, Calif. 92037.

 ISSN: 0041-1345.

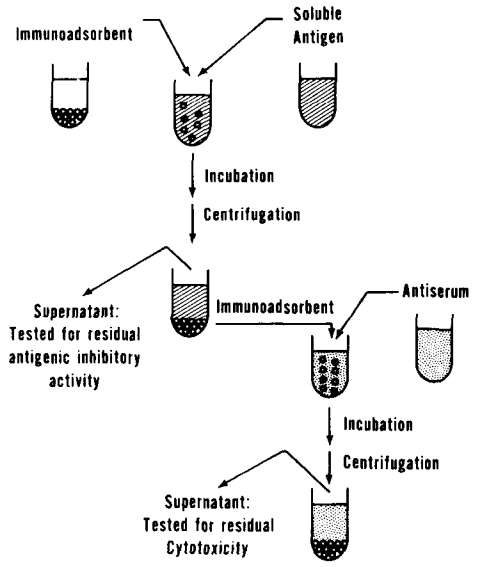

Fig. 1. Schematic diagram of immune absorption procedure.

sorbent beads removed by centrifugation, and the absorbed antisera titrated against appropriate target cells.

The results of titrating A9 allo- and xenoantisera after incubation with HLA antigen-loaded immunoadsorbents are shown in Fig. 2. The results demonstrate that the procedure provides a very sensitive means for determining the relationship between determinants recognized by different antisera. The specificity of the procedure is shown by the fact that the cytotoxic titer of HLA-A1 alloantiserum, Gaulier, was not decreased by incubation with any of the loaded immunoadsorbents, as well as by the fact that none of the antisera were adsorbed by passage over antigen-loaded immunoadsorbents of normal rabbit serum (data not shown).

The sensitivity of the method in discriminating closely associated determinants is demonstrated by the results shown in columns 1, 2, and 3 of row A. The titer of A9 alloantiserum (Fe 91/4) and A9 xenoantisera (2761-R45 and 2958-R66) was markedly decreased by incubation with the A9-loaded β_2-microglobulin ($\beta_2\mu$) immunoadsorbent compared to the titer of the antiserum absorbed by A2-loaded $\beta_2\mu$ immunoadsorbent. Thus, binding of the HLA-A9 molecule to the immunoadsorbent via antibody to determinants of the $\beta_2\mu$ chain does not result in masking of allotypic determinants of the HLA heavy chain. The procedure therefore appears to be more sensitive than the Fab'_2 blocking technique, since Fab'_2 fragments of anti-β_2-microglobulin sera block the killing of cells by HLA allo- or xenoantisera.[3]

The reactivity of two xenoantisera, both operationally monospecific for HLA-A9 by cytotoxic tests as well as by the Fab'_2 blocking and lysostrip tests, were compared. A9 xenoantiserum 2958-R66 recognizes the same determinant, or a determinant very close to, the allotypic determinant recognized by A9 alloantiserum Fe 91/4, since the alloantiserum is not absorbed by A9-loaded immunoadsorbent 2958 (Fig. 2, col-

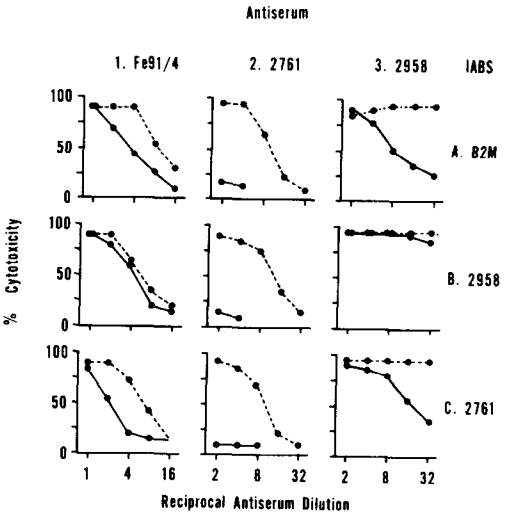

Fig. 2. Titration of adsorbed HLA-A9 allo- and xenoantisera in the microcytoxicity assay. HLA-A9 alloantiserum Fe 91/4 (column 1); xenoantiserum 2761-R45 (column 2), and xenoantiserum 2958-R66 (column 3) were tested against peripheral lymphocytes (HLA-A2,A9,B5) after incubation with immunoadsorbents loaded with HLA-A9 (solid line) or HLA-A2 (dotted line). These immunoadsorbents were anti-β_2-microglobulin (row A), HLA-A9 xenoantiserum 2958-R6 (row B), and HLA-A9 xenoantiserum 2761-R45 (row C).