SECURITY CLASSIFICATION OF THIS PAGE

(2)

REPORT DOCUMENTATION PAGE

DTIC FILE COPY

Form Approved OMB No. 0704-0188

DTIC ELECTE JUL 31 1990 B

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHOR... ULE	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
IER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
AD-A224 855	
6a. NAME OF PERFORMING ORGANIZATION: Food & Drug Administration; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): 12420 Parklawn Drive, Rockville, Maryland 20857	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: Army Project Order No. 86PP6852
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
0603105A	3M2-63105DH29	AD	021

11. TITLE (Include Security Classification)

(U) Test of Idiotype Manipulation with Monoclonal Anti-T4 Antibodies as a Potential Vaccine for AIDS

12. PERSONAL AUTHOR(S)
Suzanne Epstein

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 9/15/86 TO 4/30/90	1990 April 26	46

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	03		~~RA 1,~~ Retrovirus, Helper-T cell, Antigens, Virology
06	13		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Development of a vaccine for AIDS has proven difficult, and so alternative approaches such as idiotypic manipulation have been suggested. As applied to AIDS, this approach would involve immunizing with an anti-CD4 antibody resembling gp120 to induce anti-idiotypic antibodies which would bind to gp120. The CD4 binding site on gp120 is conserved. Thus, an idiotypic AIDS vaccine would have the advantages both of safety and of broad protection against all variants. Induction of anti-HIV immunity has been reported using anti-Leu3a, and this result has led to testing in humans, so further information about this approach is needed.

(continued on back)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION	
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL: Mary Frances Bostian	22b. TELEPHONE (Include Area Code): 301-663-7325	22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

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TEST OF IDIOTYPE MANIPULATION WITH MONOCLONAL ANTI-T4 ANTIBODIES AS A POTENTIAL VACCINE FOR AIDS

FINAL REPORT

SUZANNE EPSTEIN

APRIL 26, 1990

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

ARMY PROJECT ORDER NO. 86PP6852

Food and Drug Administration
12420 Parklawn Drive
Rockville, Maryland 20857

FOREWORD

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____For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institute of Health.

Suzanne Epstein 7-20-90
PI Signature Date

Bibliography of publications receiving support from this contract

Misplon, J.A., R.M. Kindt, J. P. Reeves, L. Harvath, L. J. Rubinstein, and S. L. Epstein. Induction of antigen-specific immunity by anti-idiotypic antibodies: Isotype expression in responses and potency of induction by monoclonal anti-idiotopes. In: Proceedings, Idiotype Networks in Biology and Medicine Congress, Gennep, the Netherlands, 17-20 April, 1989, Amsterdam, Elsevier Science Publishers, 1989, in press.

Misplon, J.A. J.P. Reeves, L. Harvath, L.J. Rubinstein, and S.L. Epstein. Induction of antigen-specific immunity with monoclonal anti-idiotypic antibodies *in vivo*: differences in potency and comparison of immunochemical properties. European Journal Immunology, 19: 2361-2365, 1989.

Submitted:

Reeves, J. P., D. Buck, I. Berkower, D. Murphy, and S.L. Epstein. Anti-Leu3a induces combining site-related anti-idiotypic antibody without inducing anti-HIV activity.

Abstracts:

Immunization with monoclonal anti-idiotypic antibodies in vivo: dramatic differences in potency. S.L. Epstein, L. Harvath, J.P. Reeves, L.J. Rubinstein, and J.A. Misplon. Presented at FASEB meeting in New Orleans, LA., March 1989.

Misplon, J.A., R.M. Kindt, J. P. Reeves, L. Harvath, L. J. Rubinstein, and S. L. Epstein. Induction of antigen-specific immunity by anti-idiotypic antibodies: Isotype expression in responses and potency of induction by monoclonal anti-idiotopes. Presented at conference "Idiotype Networks in Biology and Medicine" in Gennep, The Netherlands, April, 1989.

Personnel receiving contract support

Prior to 1/20/87, no staff supported.
Prior to 4/24/88, no full time or professional staff supported.

Lisa Shueh, Biological Aid, 12-15 hours/ week 1/20/87-5/12/89

Dr. James P. Reeves, Staff Fellow, FDA, CBER, DBB 4/24/88-4/24/90
Full time

Rachel Kindt, (college student), Biological Aid Summers of 1987 and 1988

BACKGROUND

In vivo treatment with idiotypic or anti-idiotypic antibodies has been shown in a number of systems to be capable of inducing antigen-specific immunity, without exposure of the host to antigen. Protective immunity to a number of pathogens has been induced by this means in experimental systems.

This approach can be applied to the problem of development of a vaccine for AIDS. A variety of evidence demonstrates that the CD4 molecule on a subset of human T lymphocytes and certain other cell types functions as a receptor for HIV, and interacts with the viral envelope glycoprotein. Thus, anti-CD4 antibodies include some complementary to the viral binding site, and might be capable of inducing antibodies specific for HIV itself. Our goals were to investigate this possibility, and also to study idiotype induction in a model system, to provide background for the studies in the HIV system.

RESEARCH SUMMARY

The research projects fell into two areas: studies of anti-CD4 antibodies as possible inducers of anti-HIV immunity, and studies of idiotypic induction in the Ia.7 model system, in order to better understand the nature of and requirements for such responses.
These areas will be discussed separately. In both areas, we have used the approach of deriving monoclonal antibodies in order to resolve fine specificities which may include rare ones, and in order to have reproducible reagents in large supply.

Anti-CD4 system.

In our investigation of anti-CD4 antibody as a possible idiotypic vaccine for AIDS, we have taken two approaches. We have studied existing monoclonal anti-CD4 antibodies for their potential, and we have also studied antibody responses to CD4 and derived new monoclonal antibodies.

Studies of existing anti-CD4 mAbs.

1. Early in the course of the grant period, we studied anti-idiotypic antibodies to OKT4A, a monoclonal anti-CD4 antibody selected because it competes with HIV envelope for binding to CD4. We derived a new set of anti-idiotypic monoclonal antibodies, using a cross linking ELISA for screening. Representative behavior of the cross-linking assay is shown in Fig. 1. Idiotypic specificity of the anti-idiotypic

monoclonal antibodies in the cross-linking ELISA is shown in Fig. 2.

2. The next objective with regard to these anti-idiotypes was testing them for possible reactivity with HIV envelope components. We investigated a variety of techniques. It should be pointed out that no detection system is satisfactory for this purpose unless the viral material used is reactive with CD4, since only that one epitope is an expected site for reactivity in this system. Thus, many sources of viral antigen which contain some envelope sites are nonetheless inappropriate, and a negative result in such an assay is uninformative.

We tested the anti-idiotypic antibodies by ELISA on commercial HIV-coated plates, by Western blotting with commercial HIV strips, and by fluorescence on infected H9 cells. No positive results were obtained, and so those anti-idiotypes probably do not react with HIV. In addition, none of the anti-idiotypes was able to neutralize HIV plaque forming activity in assays performed in the lab of Dr. Ira Berkower.

3. Another important candidate we considered is anti-Leu3a, described in the literature as having the ability to induce anti-HIV responses. Inconsistent results of reports concerning anti-Leu3a were suggested to be due to differences in immunization protocol. Therefore, we immunized mice with KLH conjugates of Leu3a and OKT4A, and also of MOPC-21 as an *in vivo* control following the protocol of Dalgleish, et. al. (Lancet, 2: 1047, 1987). Sera were tested by cross-linking ELISA for anti-idiotypic activity, by direct binding ELISA for binding to gp120 or gp160, and by inhibition of binding to T cells by flow cytometry for combining site related anti-idiotypic activity. The anti-idiotypic responses to anti-Leu3a and OKT4A were stronger than those to MOPC-21, as measured by cross-linking ELISA. Sera were also tested for neutralization of HIV plaque forming activity assay by Dano Cosenza-Murphy and Dr. Ira Berkower.

Results showed that while strong anti-idiotypic responses (Fig. 3) with considerable combining site-related activity (Table 1) were present, there was no specific binding to gp160 (Fig. 4) or gp120, and no neutralization (Table 2). Thus, we do not confirm the results of Dalgleish, et. al. Our own results emphasize the importance of *in vivo* controls. For example, in some assays immune sera bound to gp120 somewhat more than did preimmune sera, but this was true with mice receiving MOPC-21, not just anti-Leu3a.

4. We initiated investigations of some additional mAbs to the gp120 binding site of CD4, which were provided by Becton Dickinson: L77, L197, and L201. These antibodies were conjugated to KLH and used to immunize mice. The sera were tested for anti-idiotypic activity by cross-linking ELISA. The time course of typical responses to each of the three idiotypes is shown in Fig. 5. Sera from week 7 of immunizations with L77 and L197 were then tested by flow cytometry for ability to inhibit binding of biotinylated idiotype to CD4 on T cells (Table 3). Inhibition was seen with homologous antibody but not with the other two antibodies. Mean titers were 1:200-1:250.

Sera from mice immunized with L201-KLH will be analyzed next. Also, potential HIV reactivity of all sera will be tested, though preliminary results suggest that none will bind HIV.

Studies of anti-CD4 responses and new mAbs.

A major direction in the CD4 project is the development of our own panel of anti-CD4 monoclonal antibodies, with the hopes that one or more of them may prove to be candidates for idiotype manipulation. Given the results of our studies in the Ia.7 model system, it is clear that clones can differ dramatically in their ability to induce antigen-specific immunity, even when they are sterically indistinguishable in the sites they recognize. For this reason, we would like to have multiple monoclonal antibodies available which recognize CD4 and additionally which compete with viral envelope for binding to CD4. Only a subset, perhaps a small subset, of antibodies with promising properties in various assays would turn out to have the necessary structure and in vivo immunogenicity.

5. To aid in screening sera and hybridoma supernatants, we established two assays for detection of anti-CD4 antibodies. First was a CD4 ELISA binding assay using recombinant soluble CD4 obtained from Drs. Daniel Capon and Timothy Gregory, Genentech. Typical results are shown in Fig. 6. Known monoclonal antibodies such as OKT4 and anti-Leu3a can be readily detected, while control antibodies such as an isotype-matched myeloma protein do not bind.

However, when the ELISA on plates coated with recombinant soluble CD4 was compared with flow cytometry on human T cells, differences were noted. Results revealed that at least one antibody (L197) was readily detected by binding to cells (Fig. 7a, direct binding to MOLT-4; Fig. 7b, inhibition of gp120 binding to MOLT-4) but was detected very poorly by ELISA (Fig. 8). This finding led us to abandon screening of fusions by CD4 ELISA, and to use instead cell surface testing. On the other hand, there are many antibodies detected poorly by binding to cells and well by ELISA; presumably these represent antibodies to sites that are not exposed on the cell surface and therefore are not of direct interest.

Our current version of cell surface CD4 binding uses MOLT-4 as the positive cell lines (CD4+ T cell) and 12E1 as the negative control cell line (CD4- T cell). Representative binding curves for control antibodies are shown in Fig. 9. This should be an improvement over use of the B cell line VDS-O used previously, because fewer antibodies to irrelevant sites would be classed as specific in the first screening.

6. We developed several assays for detection of gp120 binding to CD4. We first used an ELISA for gp120 binding to solid phase recombinant CD4. This assay was satisfactory for some purposes, but has limitations discussed below. Therefore, we switched to detection of gp120 binding to human T cells by flow cytometry. Bound gp120 is detected by a rabbit antiserum kindly provided by Dr. Gale Smith, MicroGeneSys. This method gave excellent results, but is impractical for screening large number of samples from fusions. We therefore adapted the method for fluorescence measurements on the Pandex Screen Machine, which permits microtiter plate

assays of large numbers of samples. Detection by this method of gp120 binding to various cell lines is shown in Fig. 10, and for the two cell lines now used in routine screening in Fig. 11.

7. Once detection of the binding of gp120 to human T cells could be measured, an inhibition version was used to apply the assay to epitope mapping of anti-CD4 mAbs. The inhibition assay correctly identifies standard mAbs as gp120-site related. For example, in Fig. 12, anti-Leu3a inhibited while OKT4 and MOPC-21 did not. This assay was therefore used for characterization of mAbs.

8. Several types of immunizations were performed in the effort to generate a new panel of mAbs directed to the gp120 binding site of CD4. Initially we used recombinant soluble CD4 as the immunogen, on the theory that use of pure antigen would give more efficient generation of the desired mAbs. However, this approach induced multiple mAbs which bound soluble CD4 but not human T cells. Presumably these antibodies recognize structures which are inaccessible on T cells. We tried giving soluble CD4 and whole T cells alternately, and sera from such immunizations will be analyzed further.

We have now switched to immunization with whole T cells. This form of antigen favors induction of immunity to the outer domains containing the desired site. Specificity thus resides in the screening and not in the immunization. In the future we hope to use other CD4 constructs such as CD4-Ig as immunogens, to regain the advantage of using a purified immunogen while retaining good immunogenicity of the gp120 binding site.

The Ia.7 model system

Monoclonal antibody 14-4-4 recognizes epitope cluster I of the mouse alloantigen Ia.7, and is a prototype clone representing the dominant idiotypic family in the response to this antigen. This system displays potent induction of antigen-specific immunity by anti-idiotypic antibodies, without exposure of the responders to antigen.

1. In order to better understand the basis for this induction and what features of anti-idiotypes were important for induction, we derived a panel of monoclonal anti-idiotypes to 14-4-4. Nine monoclonal anti-idiotypes were derived using a cross-linking ELISA for screening; anti-idiotypic specificity of their binding is shown in Fig. 13.

The nine were characterized for a variety of immunochemical properties, as well as for *in vivo* inducing ability. A summary of anti-idiotype properties is given in Table 4, while representative supporting data follow.

2. The anti-idiotypes were characterized for competitive binding to the original idiotype 14-4-4, to determine whether they see the same or different idiotypic sites. Results are shown in Fig. 14. The sites recognized by the various mAnti-Ids could not be distinguished into independent clusters by this steric assay since all were inhibited by the others, though not always reciprocally. The lack of reciprocal inhibition may reflect allosteric effects.

The nine anti-idiotypes all inhibited binding of 14-4-4 to Ia.7-positive cells (see Table 4), indicating that the sites they recognize are in or sterically near the antigen-combining site.

3. The anti-idiotypic antibodies have also been tested for cross-reactivity on a panel of monoclonal anti-Ia.7, epitope cluster I antibodies to test for their fine specificity. One of them (17.4) cross-reacted on clones other than 14-4-4 (Fig. 15). The other anti-idiotypes did not cross-react on any of the panel (Fig. 16).

4. All of the nine anti-idiotypes were used in the form of KLH conjugates in attempts to induce I-E specific immunity *in vivo*. Results of flow cytometry tests are summarized in Fig. 17 and Table 4. By either the criterion of mean fluorescence or of fraction of individuals responding, 17.1 and 21.3 are the strongest inducing agents, and 17.2 and 17.5 are the weakest inducing agents. The rank order based on the means of anti-I-E measured was:

$$17.1 > 21.3 > 17.4 > 21.1 > (17.3 = 21.2 = 21.7) > (17.2 = 17.5)$$

Both tests of individual sera in multiple assays and repeat immunization groups in independent sets of mice showed reproducibility of the results.

5. A time course study is shown in Fig. 18. After treatment with 17.4-KLH, mice produced a detectable antibody response by 7 to 10 days. Responses remained high for approximately 10 weeks without further boosting, and then declined to near baseline levels by 15 weeks. The above time course study employed a strong inducer. We repeated such studies with a poor inducer (17.5), in case we were missing the response due to an altered time course. There was no activity at any time point.

6. The isotype distribution of the induced antibody was analyzed by use of IgG1 and IgG2a specific developing reagents in the flow cytometry experiments, and by use of cytotoxic assays to detect possible IgM. Representative results are shown in Fig. 19 for responses to 17.4. Most I-E-specific antibody was IgG1, as expected from previous studies. Occasional animals produce significant IgG2a antibody. As shown in Table 4, no cytotoxic activity was detected in most sera. In immunizations with antibodies 17.1, 17.2, 17.3, and 17.5, no lysis above 15% was seen, which is not significantly different from the complement control. The few sera that showed low levels of cytotoxicity in most cases contained IgG2a (compare Fig. 19), and did not show 2-ME sensitivity (data not shown). Thus, with limited testing no evidence for IgM was found.

7. Sera from immunized animals were also tested by ELISA for antibody reactive with the anti-idiotype used for immunization. The

reactive material can be termed Ab3. Levels of total Ab3 were measured for serum pools (Fig. 20) and for individual sera. When normalized for assay sensitivity by comparison to the 14-4-4 control, all nine mAnti-Ids induced comparable levels of Ab3, showing that all immunizations were successful technically and biologically.

8. Dose response testing with single doses of 17.4-KLH showed that 40, 20, and 10 μg doses all gave equivalent responses. Doses of 1 μg and 10 ng were unable to induce a response (Fig. 21). We questioned whether the weak inducer pattern could be overcome by a higher dose. Mice were immunized with one of the weakest inducers, 17.5, with two injections of 100 μg per dose, five times the usual dose. Most mice still failed to respond, although the minority that did respond gave quite strong responses (Fig. 22).

9. Endpoint titer in a solid phase assay such as ELISA correlates with affinity, although antibodies of quite high affinity are not well distinguished. We measured the endpoint titers for the binding of biotin-14-4-4 to plates coated with each of the mAnti-Ids, corrected for control binding of anti-κ. As shown in Table 4, there was a 100-fold range in the endpoint titer ratios obtained. There was no correlation of endpoint titer with inducing potency of the mAnti-Ids. One of the poorest inducers, 17.2, and one of the best, 17.4, had the same low endpoint titer ratios, and of the four mAnti-Ids with the higher endpoint titer ratios (0.33 to 1.00), only one was one of the strongest inducers.

10. Isoelectric focussing of the mAnti-Ids was performed by Dr. Leonard Rubinstein, Division of Bacterial Products, CBER, FDA. All nine gave distinct patterns, indicating that none of the clones are identical.

11. The populations of specific anti-Ia.7 induced by each mAnti-Id were analyzed for combining site-related idiotope structure (Fig. 23). The method used was inhibition of binding to Ia.7-positive cells, and detection by flow cytometry. Results showed that 17.1 and 21.3 induced populations inhibitable by both 17.1 and 21.3, and thus may be inducing comparable or identical antibodies. All other mAnti-Ids induced populations with distinct inhibition patterns. Thus, the mAnti-Ids interact with quite different populations within the antibody repertoire, and there are quite a few distinct ways to make a variable region related to the 14-4-4 idiotypic family and still able to bind Ia.7.

12. Antigen-specific antibody in most experiments was defined by binding to cells of B10.A(2R) but not B10.A(4R), which maps reactivity to the product of the I-E locus. To confirm that the antibody was in fact specific for Ia.7, binding was analyzed in more detail on cells from a panel of mouse strains. The sera tested were from mice treated with 17.1-KLH. As shown in Fig. 24, the induced antibody bound to cells of multiple haplotypes expressing an I-E product, mapping reactivity to Ia.7.

13. To test the possibility of internal imagery in the Ia.7 system, we wanted to immunize a species of animal that itself lacked Ia.7, epitope cluster I, and therefore would not be tolerant and could make an anti-Ia.7

response. We had previously tried rabbits, but the serology of rabbit class II and the reagents available to us made the results ambiguous. We therefore tested rats and pigs. Rats express Ia.7, epitope cluster I and so were disqualified. Miniature pigs do not express Ia.7, epitope cluster I even though other pig class II antigens were readily detectable in the same assay, confirming that the target cells contained adequate numbers of class II positive cells. Therefore, pigs were suitable for the experiment proposed above.

Pigs were immunized with 17.1-KLH and 17.4-KLH. Bleedings at various times after primary immunization and after each of several boosts were tested. There was no evidence of Ia.7-specific antibody in the sera, confirming our impression that the anti-idiotypes are not internal images. Rather, they probably induce immunity via complementarity with B cell receptors based on variable region determinants.

14. In vivo treatments have been carried out with mixtures of two monoclonal anti-idiotopes.

The mixtures used and the questions being posed were:

A. A good inducer and a poor inducer. If a good inducer and a poor inducer are combined, does the response to the good inducer get suppressed by the poor inducer? Induction was still good; there is no evidence of suppression.

B. Two good inducers. If two good inducers are combined, do the resulting antigen-binding Ab3 molecules express both idiotopes, or are two independent populations induced? Responses were high as expected. Analysis is in progress to determine whether the sites are coexpressed on the induced antibodies, or whether two different populations are induced.

C. Two moderate to poor inducers. If two moderate to poor inducers are combined, do they synergize to produce a stronger response? Results here suggested a somewhat greater activity than is seen with either antibody alone, but this requires confirmation by immunizing with the mixture and with simultaneous controls of each antibody alone, to permit statistical comparison.

15. We have pursued studies of alternative ways to present anti-idiotype *in vivo*. The idea is to avoid blocking or denaturing certain idiotopes by tradition chemical conjugation procedures. We conjugated 17.1 in the following ways:

A. Traditional glutaraldehyde-KLH conjugate.

B. Coupling to Protein A Sepharose, followed by covalent attachment with dimethylpimelimidate dihydrochloride. Since protein A binds only to the Fc region, the V region should be unchanged. Protein A should serve as a carrier.

C. Coupling to hydrazide beads, which should attach to carbohydrate chains on Fc. Again, the V region should not be changed.

D. Coupling to hydrazide beads as in C, but with a mixture of 17.1 and KLH. The idea was that hydrazide beads do not have an inherent carrier, so one was added and coderivatized.

16. Mice have been immunized with conjugates A, B, C, and D described above. The mice were bled periodically, and sera assayed for

two types of activity: total response to the variable region of 17.1, as assessed by inhibition ELISA and termed Ab3; and specific response to Ia.7, as assessed by flow cytometry on B10.A(2R) and B10.A(4R) cells. Results showed that conjugate A gave very strong Ab3 responses, while Ab3 responses to the other conjugates were barely detectable (not shown). Conjugates A and D gave detectable anti-Ia.7 activity (Table 5). We therefore pursued conjugate D in further experiments.

17. We reasoned that besides the difference in conjugation procedures, A and D differ in that conjugate A was given in complete followed by incomplete Freund's adjuvant, while the beads (D) were given in saline. We have now immunized sets of mice as follows:

A. Traditional glutaraldehyde-KLH conjugate of 17.1 with Freund's.

B. Hydrazide beads coupled with KLH and 17.1, given in saline.

C. Hydrazide beads coupled with KLH and 17.1, given with an emulsion of Freund's and saline. The emulsion was given as a second injection to the same site at the time of giving the beads. Sera from these mice will now be analyzed as above.

18. Some monoclonal anti-idiotopes induce specific anti-Ia.7 responses quite effectively, while others induce only marginal responses. One possibility was that T cell help (perhaps anti-idiotypic T cell help) was limiting in the case of poor inducers. To investigate this possibility, we have performed an experiment in which we administered recombinant IL-2 *in vivo* simultaneously with 17.5-KLH. We used the procedure and dose described by Kawamura, et. al. (*J.Exp.Med.* 162: 381, 1985), except that we immunized on day 0 (in complete Freund's adjuvant) and 3 (incomplete Freund's) as we usually do. Sera from these mice showed the presence of material able to bind to 17.5 (termed Ab3); the kinetics of the Ab3 response is shown in Table 6. When sera were tested for antigen-specific activity by flow cytometry (Table 7), there was some difference between the groups suggesting a slight enhancement of the response by IL-2, but the difference was of borderline statistical significance. Repeat experiments are planned.

CONCLUSIONS

In the CD4 system, we conclude that there is no reproducible evidence for induction of anti-HIV immunity by OKT4A or anti-Leu3a in animals producing high-titered anti-idiotype. Several additional anti-CD4 antibodies also appear unlikely as candidates. However, the variety of techniques developed over the course of this work are enabling us to analyze immunity to CD4 in various responders, and characterize their responsiveness to the relevant sites. There is still no obvious reason to think that one could not ever derive an anti-CD4 antibody with the desired properties.

In the Ia.7 model system, we have used our panel of monoclonal anti-idiotypes to demonstrate potent induction of antigen-specific immunity without exposure to antigen, and to characterize its features. The inducing ability of the different anti-idiotypes appears linked to the representation of the corresponding V regions in the repertoire. Explanation of the data does not require hypothesizing that any of them

are internal images of Ia.7, and recent evidence about V region conservation suggests that even in the clinical vaccine situation, induction by non-internal image mechanisms such as that described here can be relevant. In addition, a method of immunization with some advantages has been devised.

The type of analysis performed here has revealed a considerable amount about the manner in which the antibody repertoire is expressed and can be manipulated. It remains possible that in selected situations, idiotypic manipulation will be of practical significance in vaccination.

Materials and Methods

Fusions: Fusion technique was a modification of that described by Ozato, K., N. Mayer, and D.H. Sachs, J. Immunol. 124: 533, 1980. Lymphocyte suspensions free of red blood cells were prepared by perfusing spleens from immunized animals with tris-NH_4Cl, mincing in tris NH_4Cl, and filtering through nylon mesh. Cells from the SP2/0 cell line were washed in DMEM three times to remove FBS. 10^8 lymphocytes were mixed with 10^7 SP2/0 cells and pelleted at 1000 rpm in 16X125 round bottom tubes (Falcon). Then 200 μl of 30% polyethylene glycol (PEG), m.w. 1000 dissolved in DMEM at 40°C were added to the pellet. The tubes were mixed gently for two minutes and then centrifuged for 6 min. at 1000 rpm. The PEG was diluted away from the pelleted cells by gently adding 5 ml of 40°C DMEM without disrupting the pellet. The pelleted cells were centrifuged at 1000 rpm for 5 min, resuspended in 60 ml, and plated on macrophage feeder layers. The next day 50 μl of 4X hat media was added to each well of the fusion plates. At one week, 100 hundred μl of media was removed from each well and replace with 100 μl of fresh H-T media. 10 days to 3 weeks after fusion, the culture wells were scored for growth, fusion efficiency calculated, and culture supernatants were collected for screening of antibody production.

Biotinylation: Purified antibodies at concentrations of 0.5 to 1 mg/ml were dialyzed into 0.1 M sodium bicarbonate. Biotin-N-hydroxysuccinimide ester (Calbiochem-Behring, San Diego, CA) was dissolved in DMSO at 1 mg/ml. To 1 ml of antibody, 125 μl of biotin ester was added while gently vortexing. Reaction tubes were left at room temperature for 4 hours, and then dialyzed against phosphate buffered saline (PBS).

KLH conjugation with glutaraldehyde. Monoclonal Abs (1 mg/ml) were mixed 1:1 with keyhole limpet hemocyanin (KLH) (Calbiochem, La Jolla, CA) at 1 mg/ml, and coupled with glutaraldehyde (Sigma, St. Louis, MO) at a final concentration of 0.09% for 30 min at room temperature. The reaction was stopped with lysine (Sigma), final concentration 0.17 M. Conjugates were dialyzed against PBS.

Conjugations to beads. Covalent coupling of antibody to protein A Sepharose was performed by the method of Schneider, et. al. (J.Biol.Chem., 257: 10766, 1982). Coupling of proteins to hydrazide beads used CarboLink Coupling gel (Pierce, Rockford, IL), and was performed according to the manufacturer's instructions.

Cross-linking ELISA assay: Antibodies in PBS were bound to microELISA plates (NUNC, Reston, VA) in 100 μl/well volumes at a concentration of 0.5 μg/ml. Plates were coated overnight at 4°, then blocked with 1% fetal bovine serum (FBS, Reheis, Armor Pharmaceutical Co., Tarrytown, NY) and 0.02% sodium azide in PBS for at least thirty minutes. In subsequent assay steps the diluent was PBS containing 0.5 mg/ml bovine serum albumin (BSA; Fraction V; Boehringer Mannheim Biochemicals, Indianapolis, Indiana), 0.1% Tween (Fisher Scientific Co., Fairlawn, N.J.), and 0.02% sodium azide. All test samples, biotin conjugates, and enzyme conjugates were spun in an Eppendord microcentrifuge for 2 minutes before use. Between steps plates were washed 4 times with 0.1% Tween in PBS.

First, test samples were incubated with the plates for 1-2 hours. Then a precalibrated dilution of biotin-conjugated idiotype was added and incubated for 1-2 hours. Next, a precalibrated dilution of avidin-peroxidase (ICN, Plainview, NJ) was added and incubated for 30 minutes. Finally, ABTS substrate was added (see below). When color development was appropriate, plates were read on a Titertek Multiskan spectrophotometer (Flow Laboratories, McLean, VA) at either 414 nm.

ABTS substrate preparation: 2,2'-azinobis(3-ethylbenzthiazoline sulfonic acid) diammonium salt was prepared as a 4 mM stock solution. Just before use, this was diluted 10-fold in 0.1 M citrate buffer, pH 4. Then 15 μl of 30% hydrogen peroxide were added per 100 ml.

Competition ELISA assays: Assays were performed essentially as described above for cross-linking assays, except that unlabeled competitors were preincubated with plates before addition of the biotin conjugates for 1 hour.

Inhibition ELISA assays: Assays were performed as above for the cross-linking assay except that unlabeled competitors were preincubated for 1 hour with the biotin-conjugated anti-idiotypes before addition of this mixture to the plates.

For both the competition and the inhibition ELISA assays, data was calculated as

$$\% \text{ inhibition} = \frac{\text{maximum - experimental}}{\text{maximum - background}} \times 100.$$

Immunization of mice: For treatments with mAnti-Ids in the Ia.7 system, mice were immunized with KLH conjugated monoclonal antibodies intraperitoneally, in doses of 20 μg/mouse antibody content given twice: in CFA on day 0 and in IFA on day 3.

For responses to anti-Leu3a, OKT4A, MOPC-21, L77, L197, and L201, the protocol was that of Dalgleish, et. al. Purified antibodies were conjugated to KLH. Conjugates were precipitated with alum. Doses of 10 μg/mouse were given ip six times at one week intervals. Mice were bled at intervals until 8 weeks after the last boost.

For immunizations with beads, the beads were suspended in PBS, mixed

before drawing the dose for each single mouse into the syringe, and injected ip. If CFA or IFA was given with beads, it was administered as a separate injection but also ip and given at about the same time.

Flow cytometry for detection of anti-Ia.7 antibodies: Cell preparation and staining were performed as described by Epstein, S.L., V. R. Masakowski, S.O. Sharrow, J.A. Bluestone, K. Ozato, and D.H. Sachs, *J. Immunol.* 129: 1545, 1982. Stained cells were analyzed on an Ortho FACS Analyzer.

Cytotoxic assay: Two-stage trypan blue exclusion assays were performed as described by Sachs, et. al. (*J. Immunol.* 107: 481, 1971) using mouse spleen cells as targets and rabbit complement (Low-tox rabbit complement, #ACL3051, Cedarlane Laboratories, Ltd., Hornby, Ontario, Canada).

CD4 binding assay: 96 well Nunc immunoplates plates were coated with 100 μl/well of 1-2 μg/ml recombinant CD4 in PBS overnight at 4°C, and blocked with 125 μl/well of 1% FCS + 0.02% sodium azide in PBS for 30 minutes at room temperature. All other steps used 100 μl/well assay volume; 0.5% bovine serum albumin + 0.02% sodium azide + 0.05% Tween 20 in PBS as diluent; and room temperature. Serial dilutions of control antibodies and undiluted supernatant samples were prepared in a separate plate. They were then transferred to CD4 coated plates and incubated for 1 hr. Plates were washed 3 times with PBS + 0.05% Tween 20. An optimal dilution of biotinylated monoclonal rat anti-mouse κ was then added. After incubation for 1 hr at room temperature, the plates were washed and An optimal dilution of avidin-peroxidase (ICN, Plainview, NJ) was then added. After incubation for 30 min, the plates were washed and ABTS substrate was added. Absorbance at 414 nm was measured on a Flow Multiscan MC spectrophotometer.

Combining site anti-idiotype assay: Test sera (15 μl) were incubated with 30 μl of either biotinylated anti-Leu3a or biotinylated OKT4A for 1 hr at room temperature. The diluent was Hank's Balanced salt solution without calcium, magnesium, or phenol red and containing 1% BSA and 0.1% sodium azide (FACS buffer). Thirty μl of this mixture was added to 1 x 10^6 fresh CD4 positive MOLT4 cells in FACS buffer and incubated for 30 min on ice. The cells were washed twice in FACS buffer and stained with phycoerythrin-streptavidin (Southern Biotechnology Associates, Birmingham, AL) for 30 min on ice. The cells were washed twice in FACS buffer, fixed for 5 min in 1% paraformaldehyde buffer, and analyzed on a FACScan cell sorter with a 15 mWatt air-cooled argon laser (Becton-Dickinson) using a 4 decade log amplification of the signal. Inhibition of anti-Leu3a or OKT4A binding to cells was calculated using as maximum the mean channel fluorescence of biotinylated anti-Leu3a or biotinylated OKT4A with normal mouse serum, and as background PE-avidin alone using the formula:

$$\% \text{ inhibition} = \frac{\text{sample - background}}{\text{maximum - background}} \times 100.$$

Fluorescence assay for inhibition of gp120 binding to MOLT-4:

Details are given for the Pandex version of the assay. Flow cytometry followed essentially the same procedure, except that cells were processed in tubes, washed in 2 ml volumes, and analyzed on a FACScan (Becton Dickinson).

Fresh cultures of MOLT4 (human T cell line) and VDSO (human B cell line as a control) were washed twice by centrifugation at 1500 rpm for 10 min at 4°C in FACS buffer. Cells (1 x 10^6/well) were added to 96 well round-bottomed rigid microtiter plates. The plates were centrifuged at 1000 rpm for 5 min at 4°C, the supernatant discarded and the plates mixed on a plate mixer for 15-20 seconds. Serial dilutions of antibodies OKT4 (Ortho), anti-Leu3a (Becton-Dickinson), and MOPC-21 (Cappel) were added in 50 µl/well starting at 3 µg/ml. After 30 min incubation on ice, the cells were washed twice by centrifugation and 50 µl/well of 10 µg/ml recombinant gp120 (Genentech) was added. After 1 hr incubation on ice, the cells were washed twice and 50 µl/well of a pre-determined dilution of rabbit anti-gp120 (Gift of Dr. G. Smith, MicroGeneSys) was added. After 30 min incubation on ice, the cells were washed twice, and 50 µl/well of an appropriate dilution of FITC labeled goat anti-rabbit IgG (Miles) was added. After 30 min incubation on ice, the cells were washed and fixed for 5 min in 1% paraformaldehyde in PBS. The cells were centrifuged for 5 min at 1000 rpm at 4°C, resuspended in FACS buffer, and half the cells transferred to assay plates. Fluorescence units were measured on a particle fluorescence concentration analyzer (Pandex) at 485-535 nm.

Table 1. Inhibition of biotinylated anti-CD4 antibody binding to MOLT-4.

Biotinylated antibody (1 μg/ml)	Test serum dilution	% inhibition ± SEM** by sera from mice immunized with: anti-Leu3a	OKT4A	MOPC 21
anti-Leu3a	1/1	90 ± 1*	32 ± 15	13 ± 6
	1/50	92 ± 3*	10 ± 3	-1 ± 5
	1/250	48 ± 12*	4 ± 2	-5 ± 3
OKT4A	1/1	-1 ± 13	77 ± 7*	-15 ± 4
	1/50	-12 ± 8	74 ± 16*	-3 ± 3
	1/250	-5 ± 2	26 ± 11*	-4 ± 2

** Percentage inhibition of biotinylated antibody binding ± standard error of the mean by serum from mice at 7 weeks (2 weeks after the last injection).

* Different from sera of other immunized groups tested at the same dilution against the same biotinylated antibody ($p < 0.05$)

Table 2. Plaque assay test of neutralization of HIV by immune serum[++].

Sample	Serum dilution[+]	100 µl HIV		30 µl HIV	
		# plaques	(% Surviving virus[*])	# plaques	(% Surviving virus[*])
HIV only		189	100%	60	100%
Patient #9[**]	1/16	5	3%	4	7%
Pre-immune	1/16	160	85%	62	103%
MOPC-21[++]	1/16	182	96%	82	136%
Anti-Leu3a #1	1/16	170	90%	65	108%
#2	1/16	163	86%	99	165%
#3	1/16	212	112%	76	127%
#4	1/16	199	105%	80	133%
#5	1/16	170	90%	95	158%
#6	1/16	195	103%	69	115%
Group mean	1/16	185	98%	81	135%
OKT4A #17	1/16	203	107%	70	117%

[++] Mice immunized with KLH conjugates of MOPC-21, anti-Leu3a, or OKT4A. Immune sera tested here were taken 8 weeks after the sixth injection.

[+] All mouse sera were also tested at a dilution of 1/160, and showed no neutralizing activity.

[*] % surviving virus calculated relative to V_o (input plaques) - plaques in no serum control. Two levels of input virus were used (see column headings).

[**] Human serum from an AIDS patient, #9, served as a positive control for neutralization. This serum has a neutralizing titer of approximately 800.

Table 3. Inhibition of biotinylated anti-CD4 antibody binding to MOLT-4.

Biotinylated antibody (1 μg/ml)	Test serum dilution	% inhibition ± SEM** by sera from mice immunized with: L77	L197	L201	Preimmune
L77	1/10	100 ± 0*	20 ± 5	11 ± 4	-8 ± 18
	1/50	79 ± 19*	-14 ± 7	-17 ± 1	
	1/250	50 ± 20*	-18 ± 16	-20 ± 12	
	Mean titer	1:191	<1:10	<1:10	
L197	1/10	-1 ± 8	97 ± 0*	-13 ± 20	2 ± 5
	1/50	-18 ± 4	80 ± 9	-36 ± 36	
	1/250	-10 ± 9	33 ± 12	-10 ± 23	
	Mean titer	<1:10	1:250	<1:10	

** Percentage inhibition of biotinylated antibody binding ± standard error of the mean by serum from mice at 7 weeks (2 weeks after the last injection). Sera were tested individually, six mice per group for homologous biotin-antibody and three mice per group for non-homologous.

* Different from sera of other immunized groups tested at the same dilution against the same biotinylated antibody ($p < 0.05$)

Table 4. Properties of mAnti-Ids: isotypes, inhibition of 14-4-4 binding to I-E, inducing potency, and isotypes of induced antibody.

Clone	Isotype	% Inhibition of 14-4-4 binding*	Induced anti-I-E, # mice positive/# mice tested IgG1 (mean)+	IgG2a (mean)+	Cytotox#	Endpoint titer ratio##
17.1	IgG1,κ	98.4	6/6 (63)	2/7 (7.8)	0/5	1.00
17.2	IgG1,κ	97.4	0/5 (6)	0/5 (-6.3)	0/7	0.01
17.3	IgG1,κ	97.4	3/7 (41)	0/7 (-2)	0/7	0.33
17.4	IgG2a,κ	97.0	7/8 (79)	4/9 (31.5)	3/8	0.01
17.5	IgG1,κ	95.2	1/6 (8)	0/6 (-2.2)	0/6	0.04
21.1	IgG1,κ	97.6	3/6 (47)	1/6 (7.8)	0/5	1.00
21.2	IgG1,κ	98.2	4/6 (30)	0/6 (-0.1)	0/6	0.04
21.3	IgG1,κ	97.4	6/6 (97)	0/6 (6.1)	0/6	0.11
21.7	IgA,κ	94.6	3/6 (33)	0/6 (5.1)	0/6	0.33

* Inhibition of binding of 14-4-4 to I-E.

$$\% \text{ inhibition} = \frac{(\text{uninhibited on 2R} - \text{inhibited on 2R})}{(\text{uninhibited on 2R} - \text{uninhibited on 4R})} \times 100.$$

Inhibitors were used at 200 μg/ml, and 14-4-4 used at 3 μg/ml. Inhibition by controls 187.1 (anti-mouse κ mAb), MOPC-21, and TEPC-15, all <1%.

+ Summarizes several experiments, all using sera taken at 6 weeks. Shown are number of individuals with positive responses (2R-4R fluorescence >25) vs. number tested; mean fluorescence channel given in parentheses.

Summarizes two experiments. Sera taken at day 14 were titrated individually on 2R

targets, and called positive if lysis >20% (all were <26%). C' backgrounds were <10%, anti-MHC class I antibody, >80%, anti-MHC class II 40-60%.

Endpoint titers were measured by ELISA, using biotin conjugates of 14-4-4 and of anti-κ on plates coated with each of the nine mAnti-Ids, and avidin-peroxidase. The ratio given is the ratio of 14-4-4 endpoint titer to anti-κ endpoint titer on a given mAnti-Id plate.

Table 5. Antigen-specific responses induced by anti-idiotype conjugates*.

Group	Immunogen	Mouse	Specific anti-I-E activity** 5 1/2 wks	9 1/2 wks
A	17.1-KLH + CFA (glutaraldehyde)	1	65.7	45.0
		2	N.T.	43.9
		3	66.6	26.0
		4	70.2	38.9
		5	63.0	34.2
		9	52.9	44.1
B	17.1-protein A beads	8	28.6	-12.1
		10	N.T.	- 1.0
		11	12.4	-11.8
		13	8.3	- 5.7
		14	4.3	- 7.8
		15	6.7	- 2.1
C	17.1-hydrazide beads	12	1.8	- 7.6
		17	6.4	- 7.0
		18	2.4	- 3.8
		19	17.5	- 4.3
		20	N.T.	-10.5
		21	3.1	-37.9
D	17.1-KLH hydrazide beads	22	39.3	- 7.5
		23	16.8	- 5.2
		24	28.0	-31.9
		25	N.T.	- 0.8
		26	13.3	-11.9
		27	31.1	- 1.4
Controls:				
	Normal mouse serum		5.3	-19.4
	14-4-4 (mAb anti-Ia.7)		64.2	57.3
	14-4-4 ascites, 1:100		47.8	25.9

* Mice were immunized with various conjugated forms of 17.1 prepared as described in the text. Each dose contained 20 μg of 17.1. Mice were immunized on day 0 and day 3. All bead injections were in PBS; group A injections were in CFA (day 0) and in IFA (day 3).

** Binding to B10.A(2R) and B10.A(4) LPS blasts measured by flow cytometry. Specific anti-I-E activity represents mean channel fluorescence on 2R - mean channel on 4R.

Statistical significance of differences by two-tailed Student's t test:
A vs D, $p<.001$.
B vs D, $.05<p<0.1$.
C vs D, $p<.01$.
B vs C, $0.2<p<0.4$.

Table 6. Ab3 response to anti-idiotype treatment with or without IL-2

Week	Inhibition titer*			
Treatment**:	17.5 + IL-2		17.5 - IL-2	
Mouse:	#3	#6	#8	#14
1	0	0	0	0
2	0	0	6	6
3	54	162	6	18
4	1458	485	54	162
5	485	1458	6	485
6	1458	485	54	485
8	1458	1458	485	1458
10	1458	N.T.	1458	N.T.

* 17.5-biotin was preincubated with sera, then transferred to 14-4-4 coated plates. Titers shown are 50% inhibition titers.

** Mice were immunized with 17.5-KLH conjugated by the glutaraldehyde method, 20 μg/mouse Ig content, emulsified in CFA for the first dose on day 0 and in IFA for the second dose on day 3.

Table 7. Anti-I-E response to anti-idiotype treatment with or without IL-2*

Immunogen	Mouse	Specific anti-I-E activity** 6 weeks	8 weeks
17.5-KLH + IL-2	#2	2.1	-2.2
	#3	60.9	42.8
	#4	1.9	-2.5
	#6	90.1	101.4
	#7	3.8	-4.1
	#10	54.3	22.1
17.5-KLH alone	#1	0.4	-0.9
	#5	-5.1	-1.2
	#8	-1.8	-5.7
	#9	-5.7	-0.8
	#14	0.6	-1.2
	#17	4.5	3.5

* Mice were immunized with 17.5-KLH with or without IL-2. All components were emulsified together with CFA for the first injection on day 0 and with IFA for the second injection on day 3. All doses contained 20 μg per mouse of 17.5, and were given ip.

** Binding to B10.A(2R) and B10.A(4) LPS blasts measured by flow cytometry. Specific anti-I-E activity represents mean channel fluorescence on 2R - mean channel on 4R.

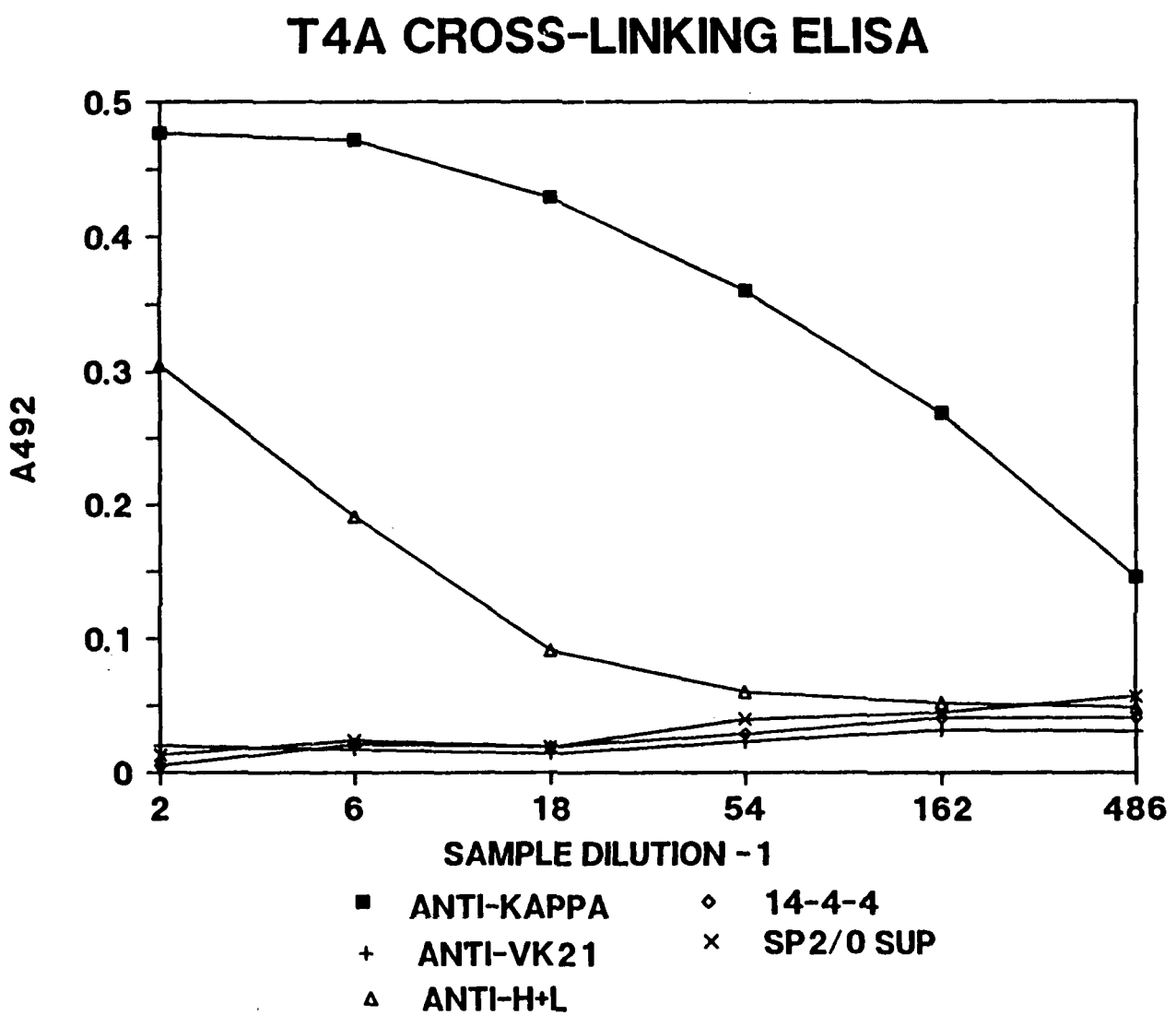


Fig. 1. T4A cross-linking ELISA, specificity controls. Assay was performed as described in Materials and Methods, using plates coated with T4A (provided by Dr. Gideon Goldstein) and biotin-conjugated T4A for detection. Test samples are: ■ , 187.1 monoclonal rat anti-mouse kappa chain constant region, cell line from Dr. Matthew Scharff via Leonard Rubinstein; + H106-150.3 culture supernatant, monoclonal rat anti-mouse Vk21, cell line from Michel Pierres; Δ pig anti-mouse IgG2a,k, affinity purified; ◇ 14-4-4, a mouse monoclonal anti-Ia.7, cell line from Keiko Ozato and David Sachs; × SP2/0 culture supernatant.

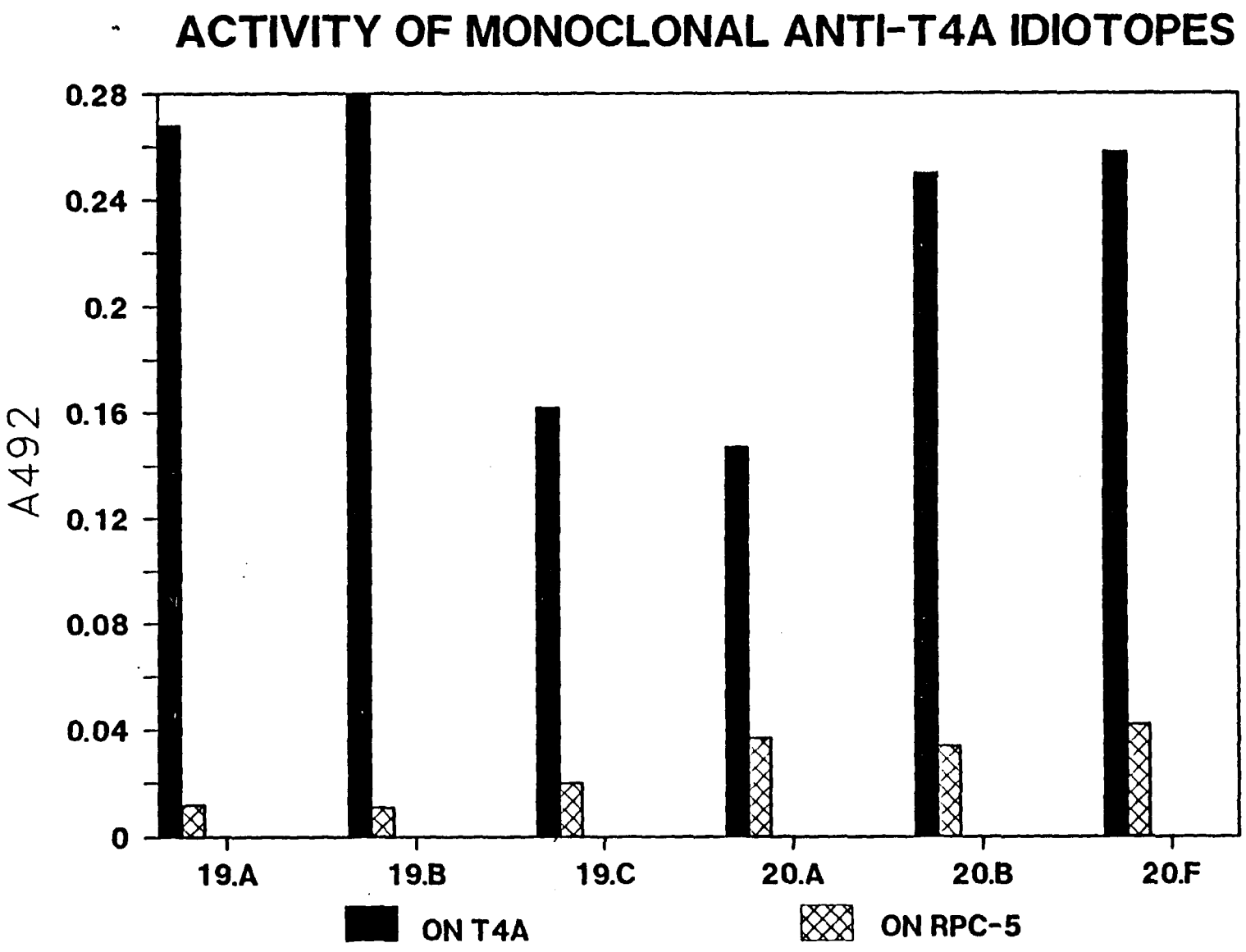


Fig. 2. Activity of monoclonal anti-T4A idiotope antibodies in the cross-linking ELISA. Solid bars show binding to plates coated with OKT4A; cross-hatched bars show binding to control plates coated with RPC-5, an IgG2a myeloma protein (Litton Bionetics, Inc.). Samples tested were culture supernatants.

Fig. 3

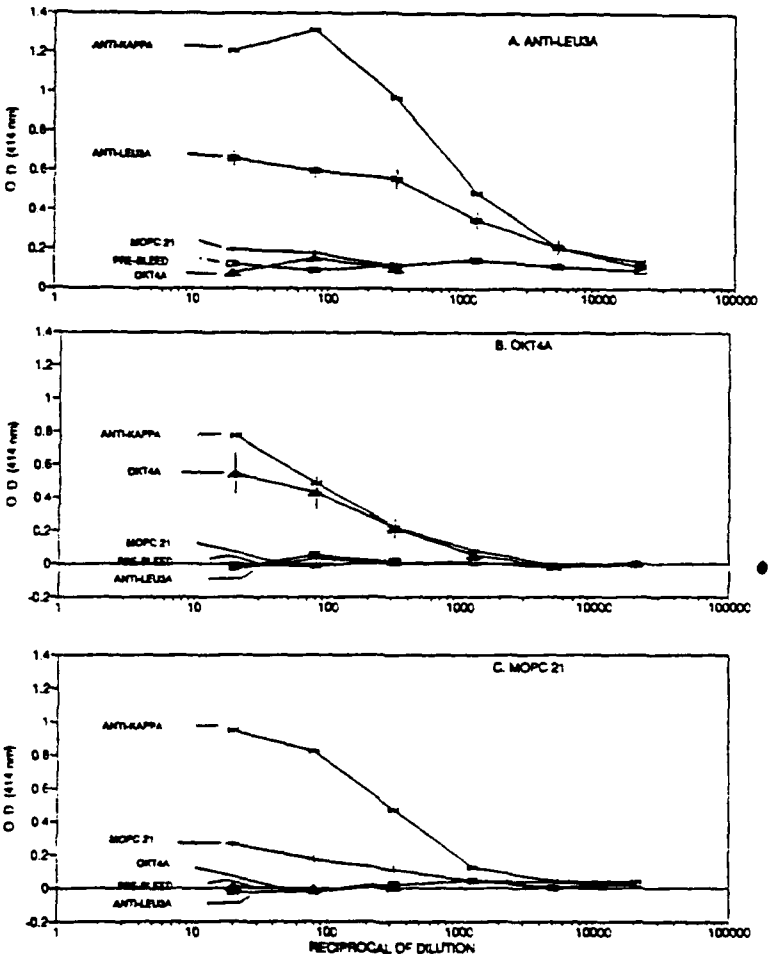


Fig. 3. Anti-idiotypic activity in sera of immunized mice detected by cross-linking ELISA. Each assay used one antibody (A. anti-Leu3a, B. OKT4A, C. MOPC-21) to coat the plates. Samples were added, followed by the same antibody in biotinylated form and then avidin-peroxidase, to detect cross-linking activity in the samples. The anti-κ control was monoclonal antibody 187.1; other curves are labeled to indicate the immunogen used to raise the sera tested. Sera from six mice immunized with the homologous antibody six times and bled 8 weeks after the last injection were titrated individually; mean OD values are plotted, +/- S.E.M. (error bars). One preimmune serum and one serum from each non-matched immunization are also shown in each case.

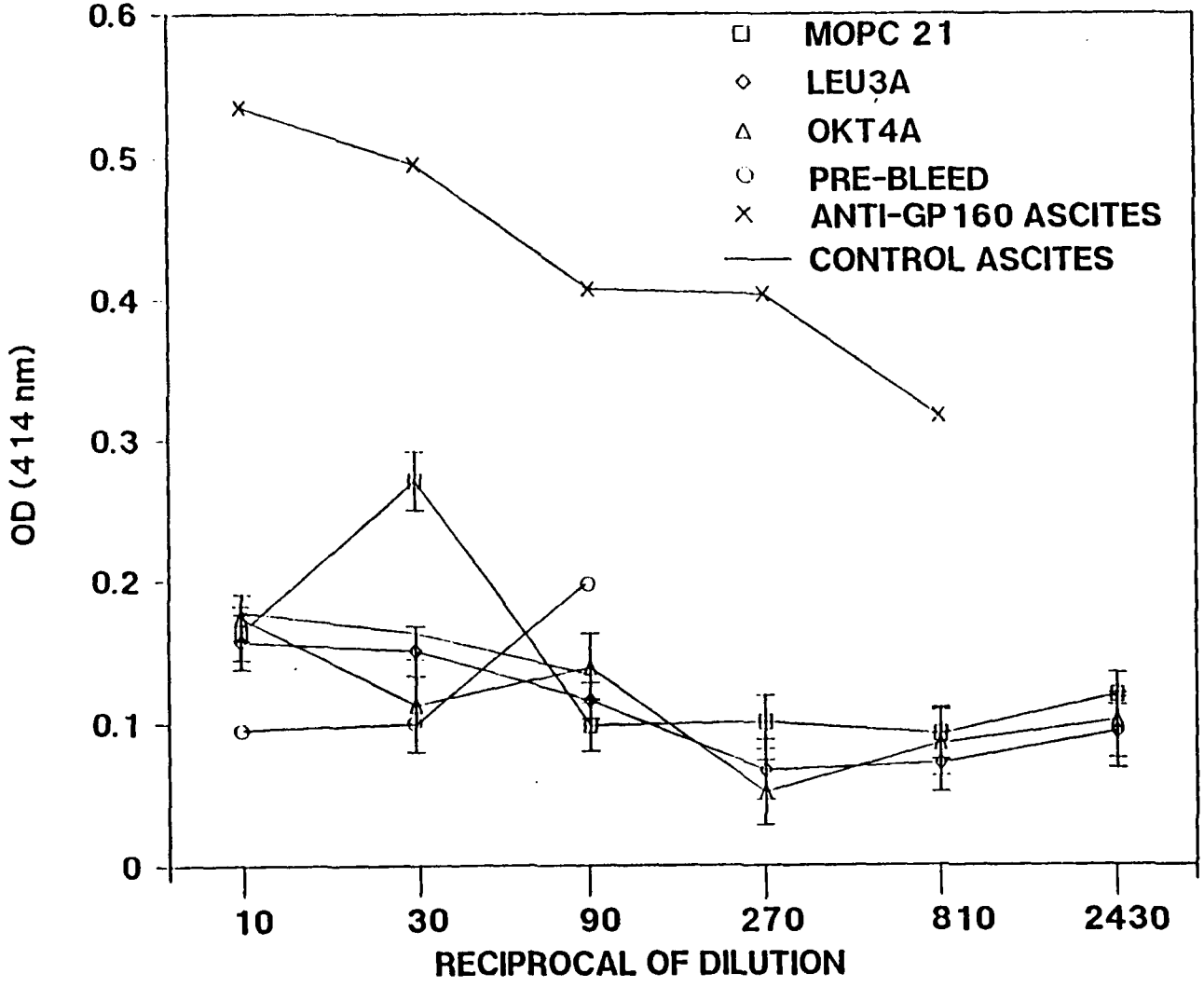


Fig. 4. Direct binding ELISA for antibodies reactive with gp160. Plates were coated with recombinant gp160 and sera were assayed as described in Materials and Methods. Sera from immunized mice taken 8 weeks after the sixth injection were titrated individually; mean OD values are plotted. Student's two-tailed t tests showed no significant difference between any two immunized groups at a dilution of 1:10 ($p>0.5$ for all comparisons, n=4 per group for this experiment, n=6 per group for other assays also showing no difference between groups). Samples: × anti-gp160 ascites; — irrelevant immune ascites; ○ individual pre-immune serum; □ group mean of mice given MOPC-21-KLH; ◇ group mean of mice given anti-Leu3a-KLH; △ group mean of mice given OKT4A-KLH.

Fig. 5

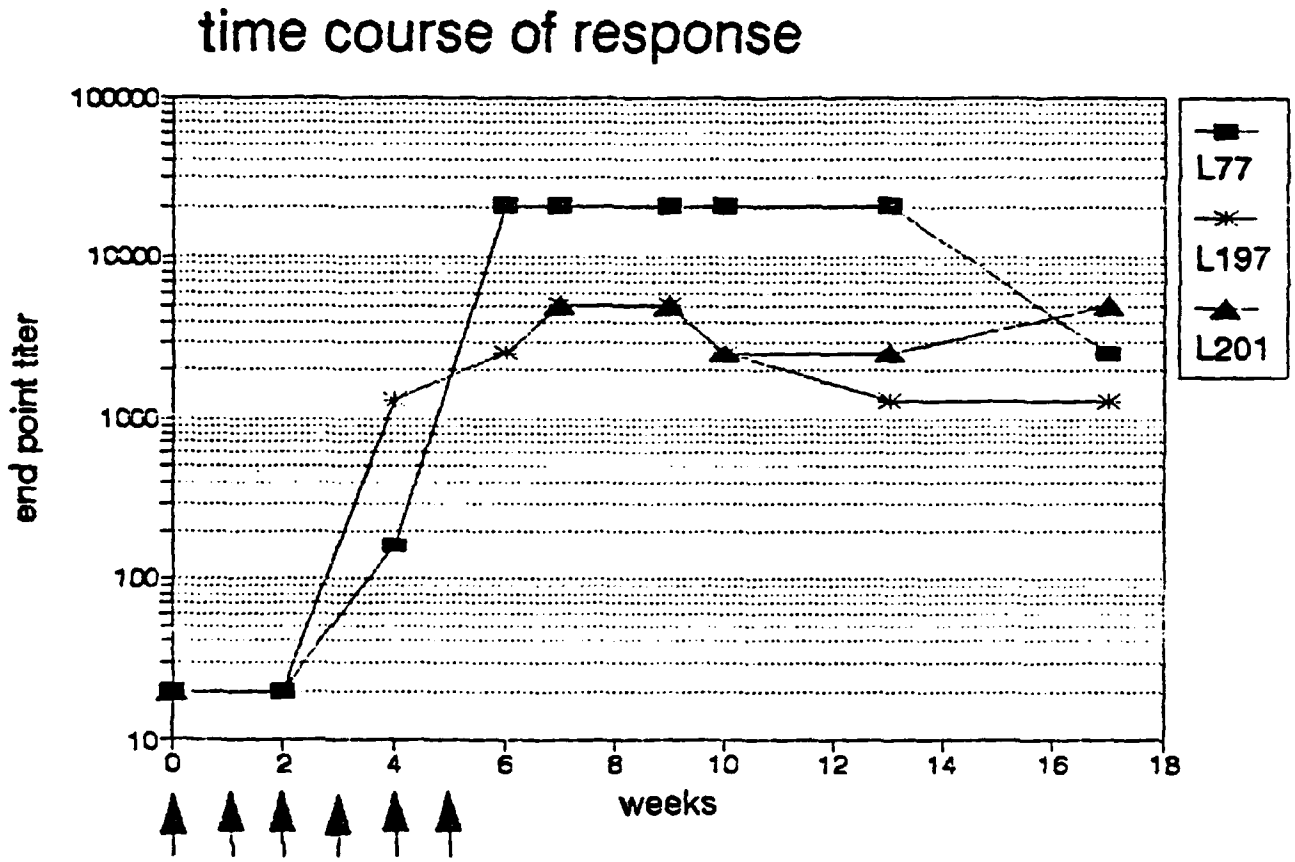


Fig. 5. Time course of the anti-idiotypic responses to L77, L197, and L201. Tested by cross-linking ELISA assays homologous to the immunogens. Representative mice followed by serum titrations at each time point. Arrows indicate times of injections.

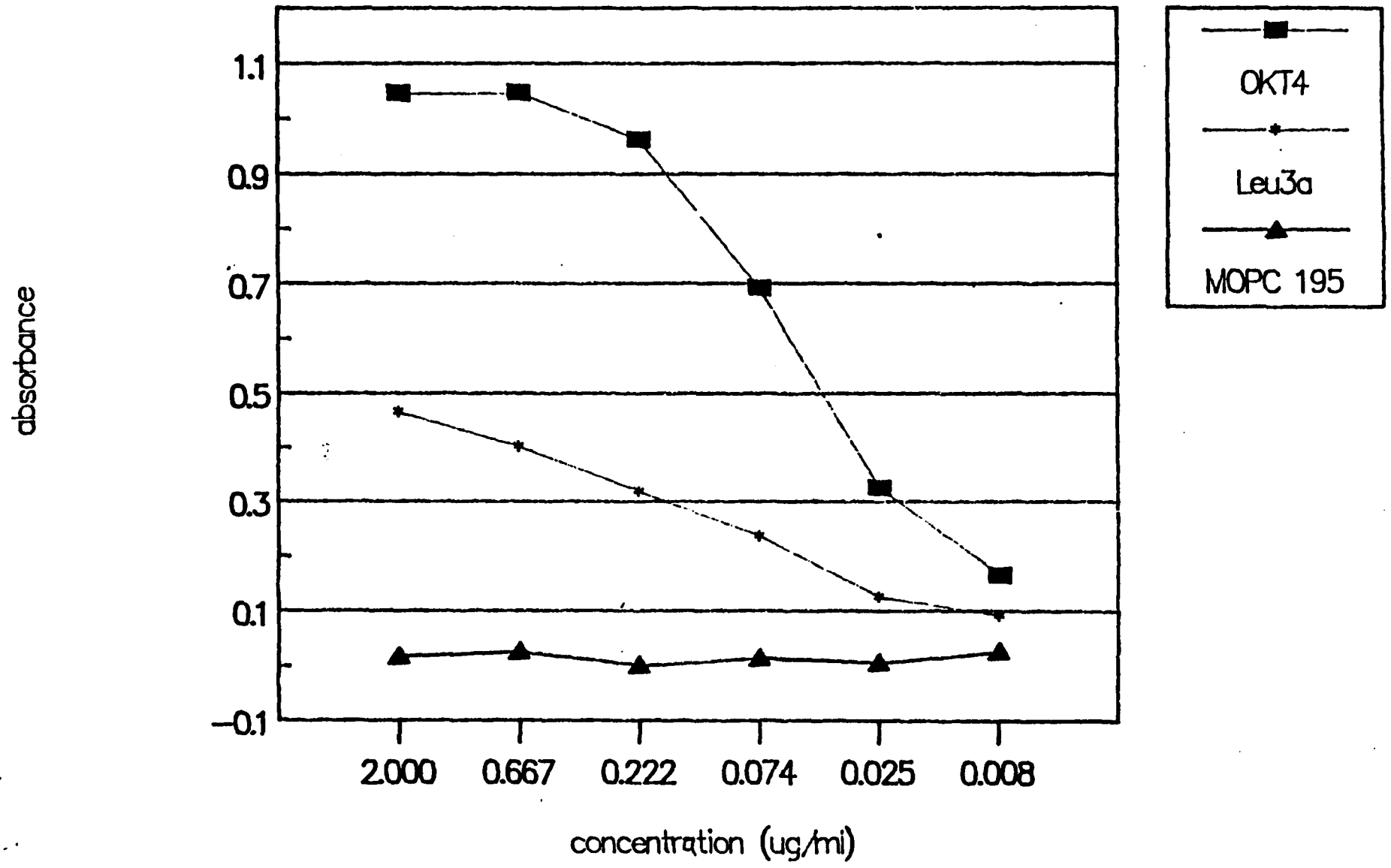
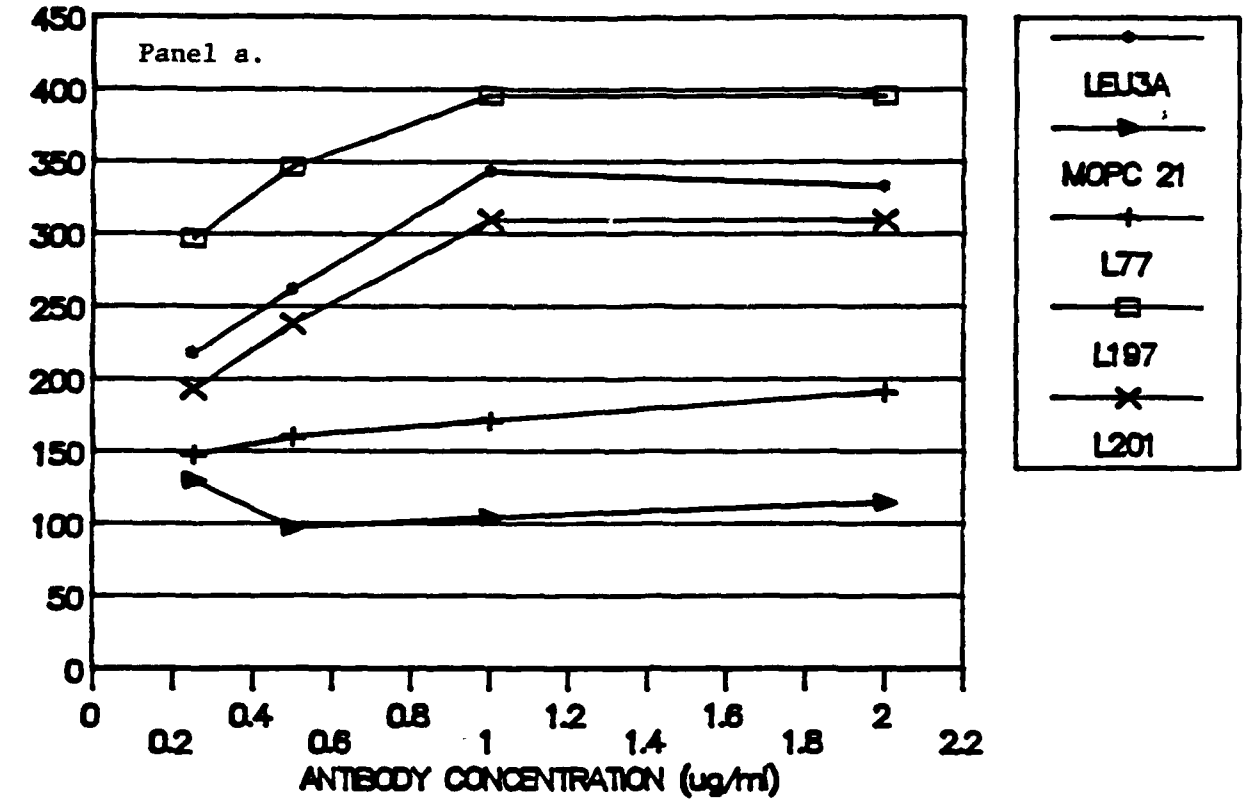


Fig. 6. Binding of monoclonal antibodies to rCD4 detected by ELISA. Control antibodies OKT4 (■), Leu3a (*), or MOPC 195 (▲) were serially diluted and tested by ELISA for binding to CD4-coated plates. Absorbance (414 nm) is plotted against concentration in μg/ml.



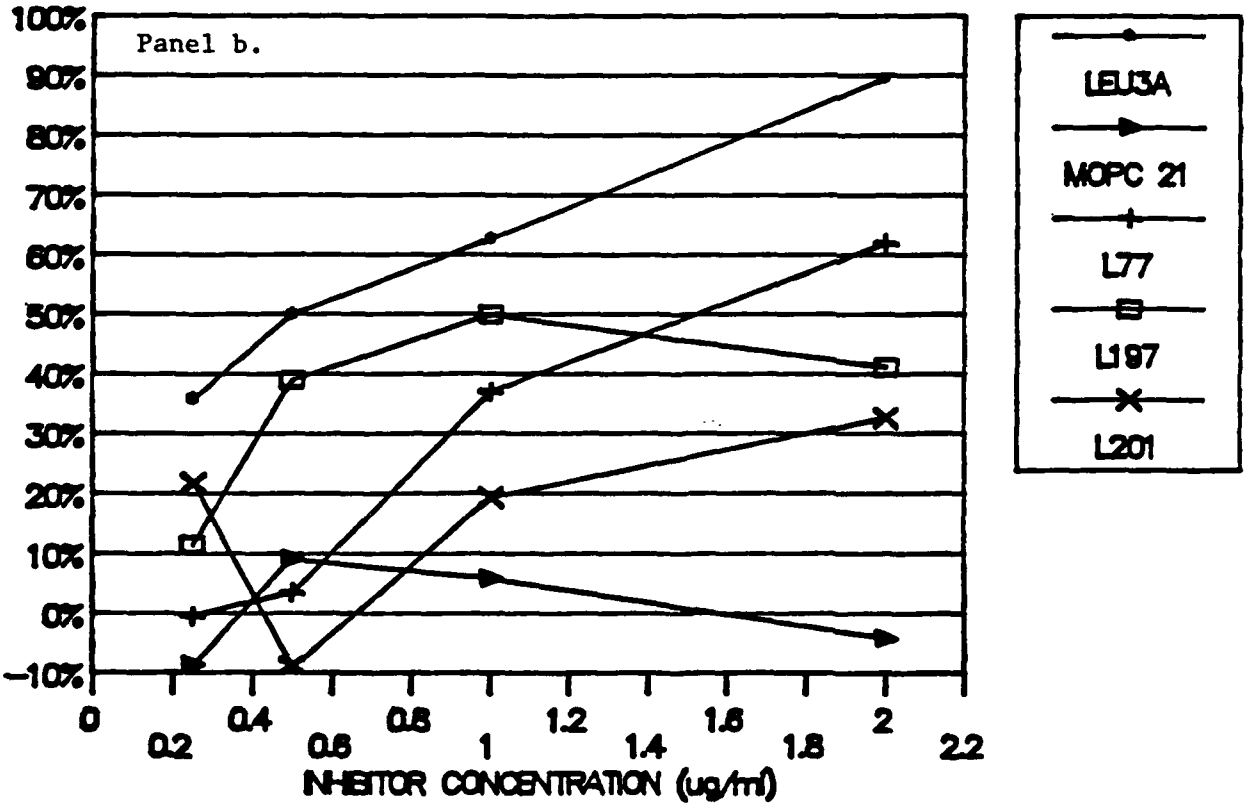


Fig. 7. Epitope mapping with anti-CD4 antibodies by flow cytometry. a. Direct binding of monoclonal antibodies to MOLT-4 cells. b. Inhibition of gp120 binding by anti-CD4 antibodies.

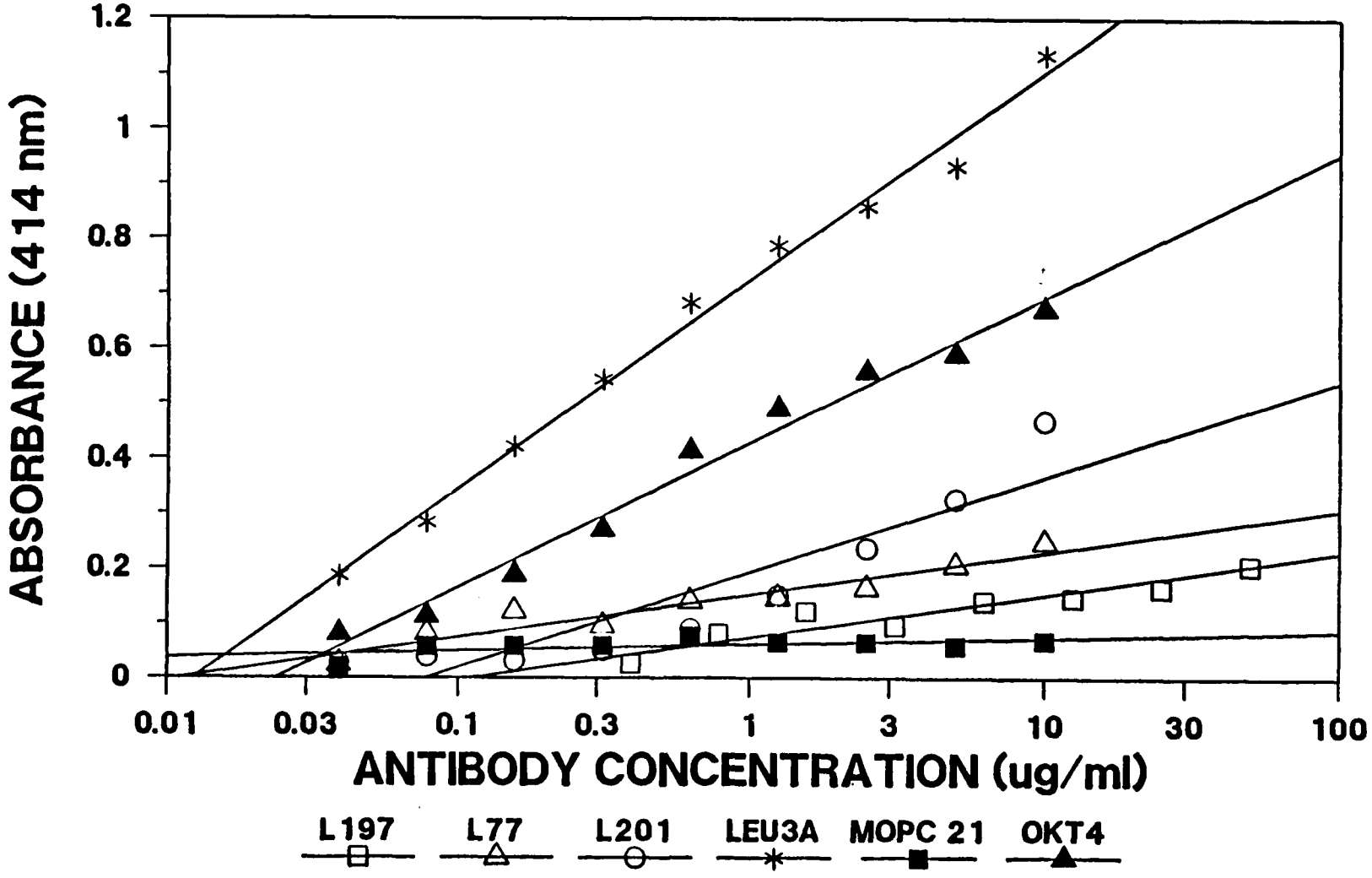


Fig. 8. Solid phase ELISA for detection of antibodies binding to CD4. Plates were coated with recombinant soluble CD4 at 1 μg/ml.

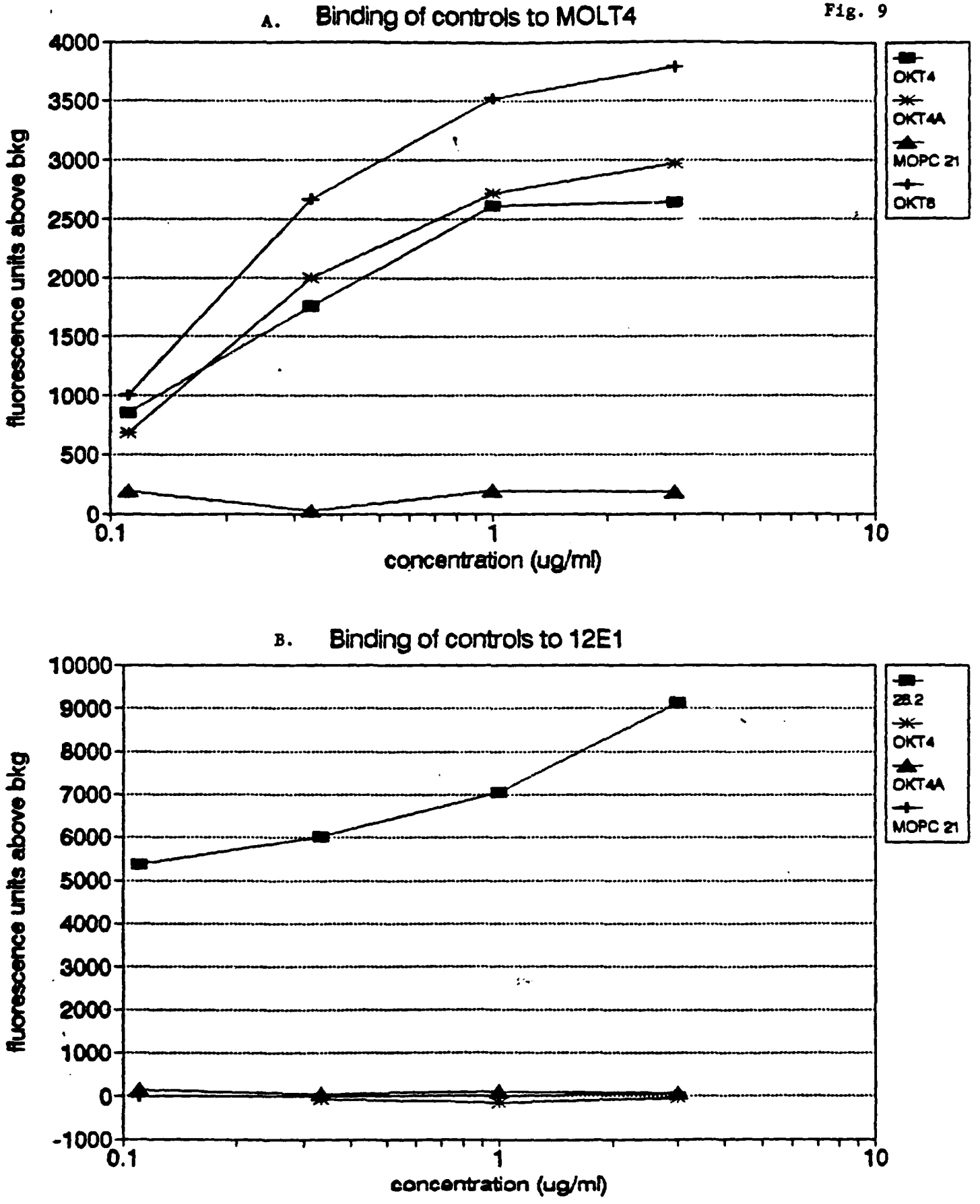


Fig. 9. Binding of monoclonal antibodies to human T cells, as detected by Pandex fluorescence assay. MOLT-4: $CD4^+$ human T cell line. 12E1: $CD4^-$ human T cell line. Clone 28.2 is a monoclonal antibody derived in the course of this project that binds to human T cells but not to CD4.

Fig. 10

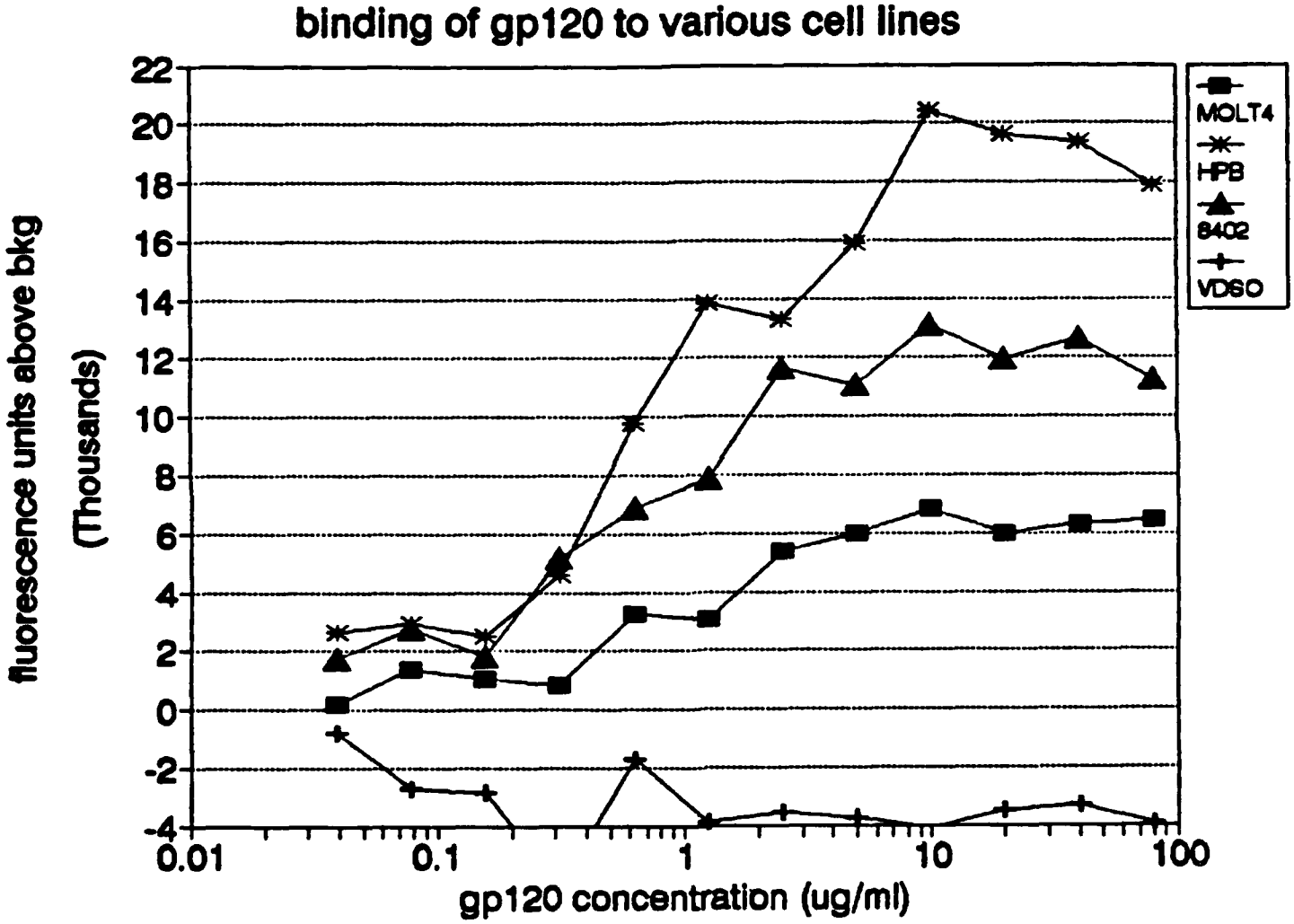


Fig. 10. Binding of gp120 to human cell lines. MOLT-4, HPB, and 8402 are human T cell lines. VDS-O is a human EBV-transformed B cell line. Bound gp120 detected with rabbit anti-gp120 followed by FITC-goat anti-rabbit Ig, as described. Fluorescence measured on the Pandex machine.

Fig. 11

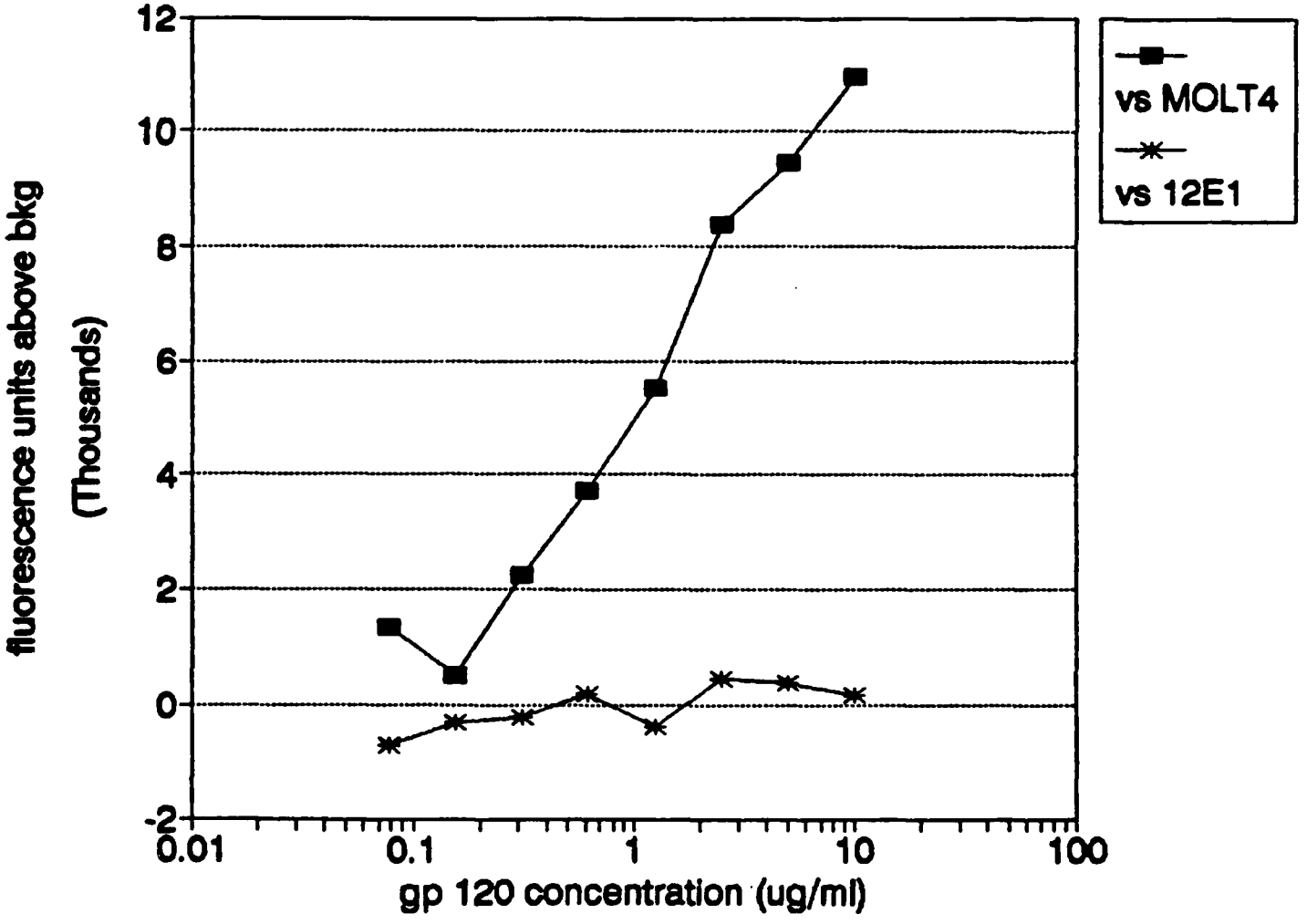


Fig. 11. Comparison of gp120 binding detected by the Pandex assay on the two human cell lines now used for hybridoma screening: MOLT-4: $CD4^+$ human T cell line. 12E1: $CD4^-$ human T cell line.

INHIBITION OF gp120 BINDING TO MOLT4

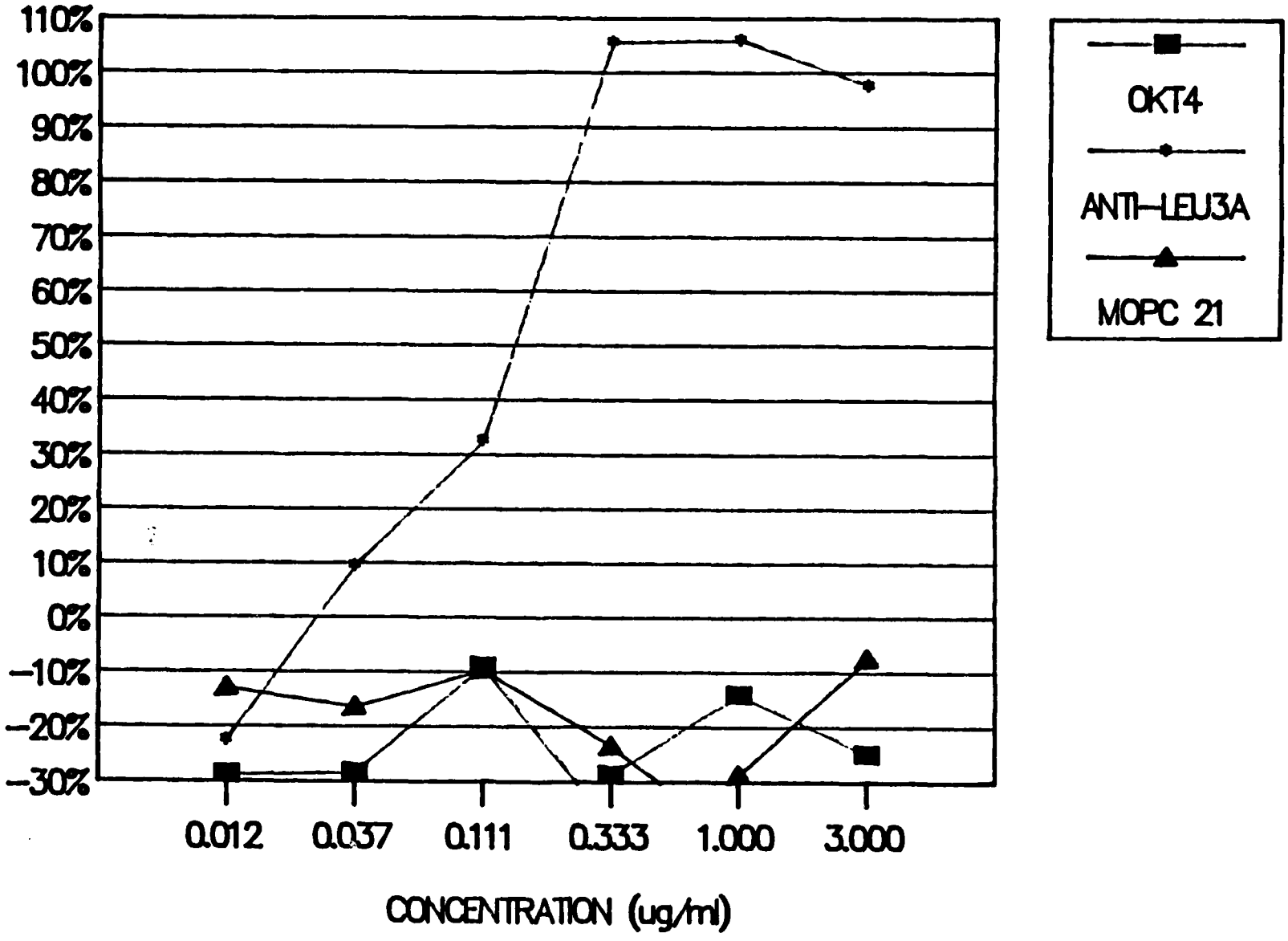


Fig. 12. Inhibition of gp120 binding to MOLT-4 cells by fluorescence assay as in Materials and Methods. Briefly, cells were incubated with anti-CD4, then gp120, then rabbit anti-gp120 serum, and then FITC-goat anti-rabbit Ig. Detection was done on the Pandex Screen Machine.

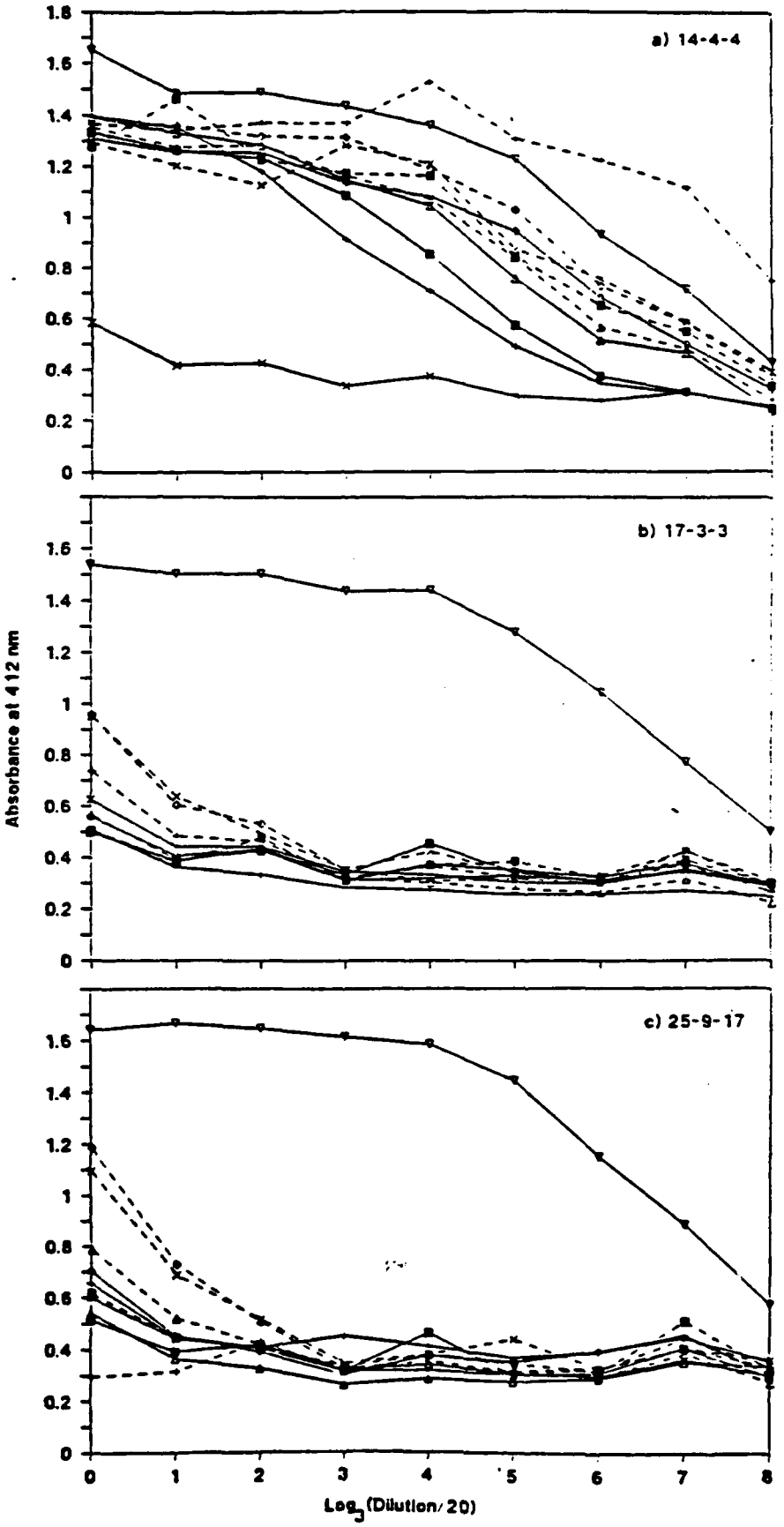


Fig. 13. Specificity of monoclonal anti-idiotopes. Biotin-conjugated anti-idiotopes were tested by direct binding ELISA on plates coated with: a) 14-4-4, b) 17-3-3, and c) 25-9-17. Bound antibody was detected with avidin-peroxidase followed by substrate reactions. Biotin-conjugated antibodies: solid lines, ■ 17.1, + 17.2, ◇ 17.3, △ 17.4, X 25-9-17, ▽ 187.1; dashed lines, ■ 17.5, + 21.1, ◇ 21.2, △ 21.3, X 21.7.

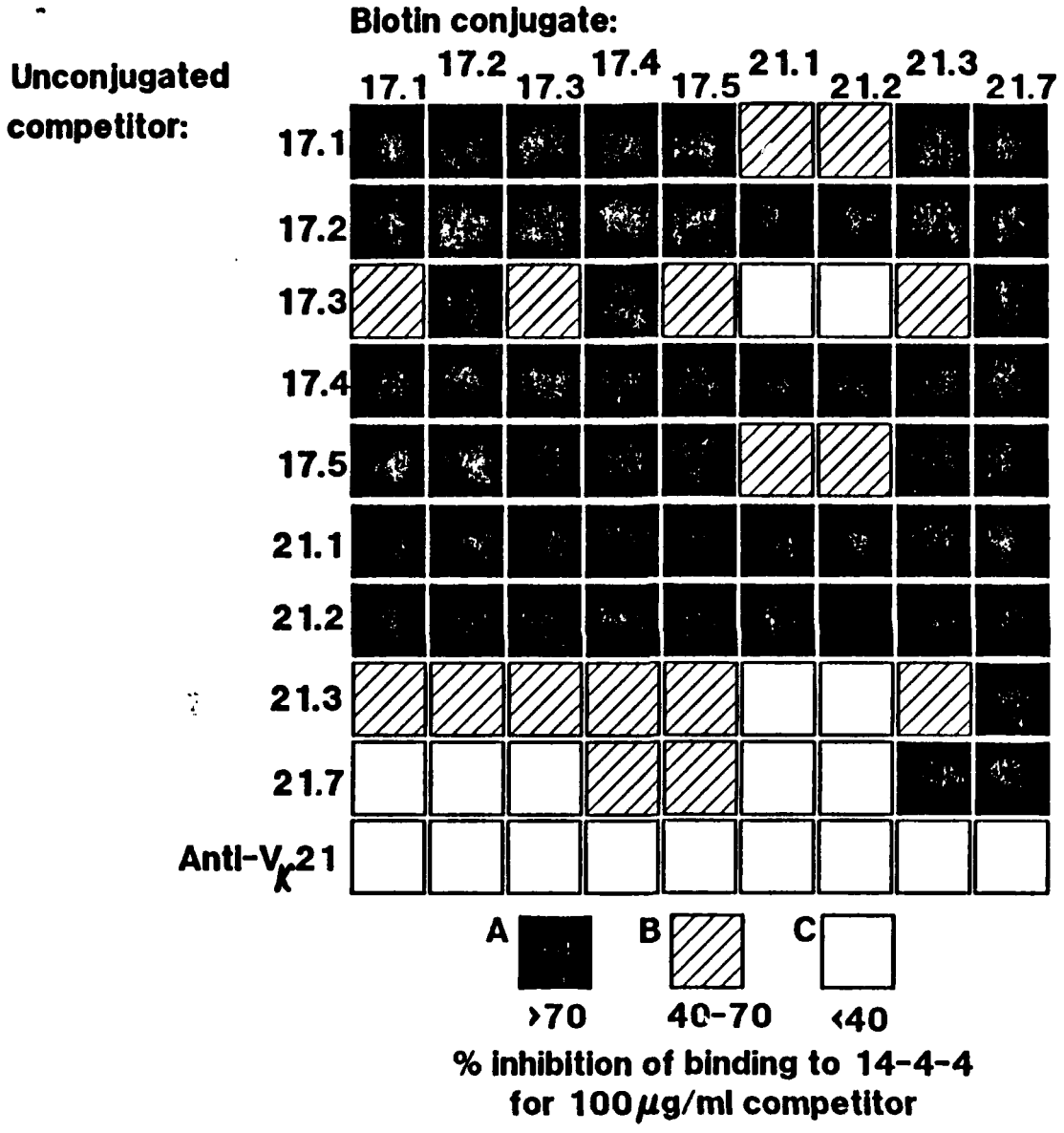


Fig. 14. Competitive binding of monoclonal anti-idiotopes. Unconjugated monoclonal anti-idiotopes were tested for ability to inhibit the binding of biotin-conjugated anti-idiotopes to 14-4-4 coated plates. Detection was by avidin-peroxidase. All unconjugated competitors were titrated; results at the highest concentration tested are shown. Anti-Vκ21 is a control monoclonal antibody binding to the variable region framework of 14-4-4.

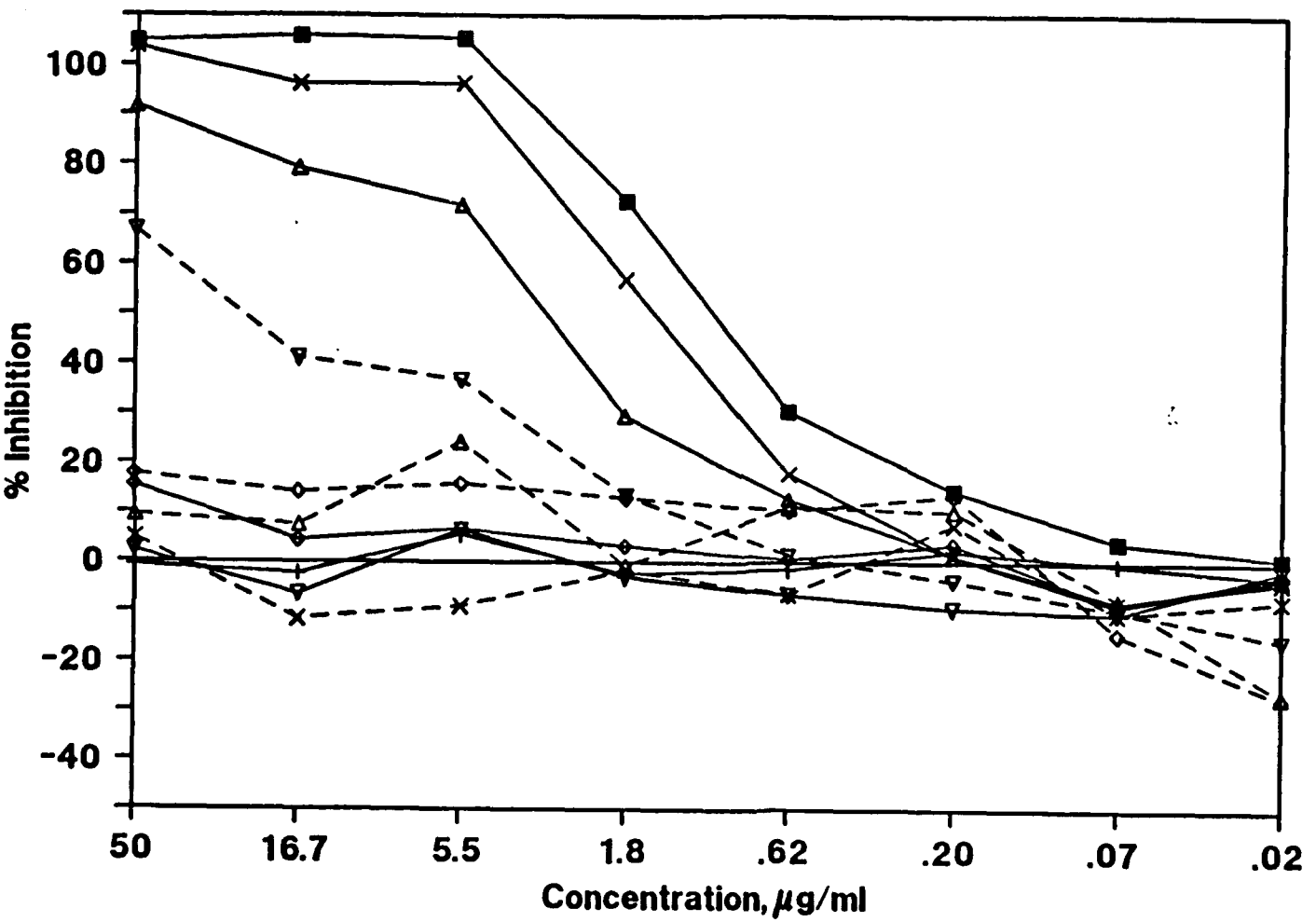


Fig. 15. Cross-reactivity of 17.4 with anti-Ia.7 mAbs. Unlabeled anti-Ia.7 antibodies were preincubated with the biotin-conjugated 17.4. The mixtures were then transferred to 14-4-4 plates, and bound mAnti-Id detected with avidin-peroxidase. Solid lines, ■ 14-4-4, + RPC-5, ◊ 81.B, Δ 81.C, X 81.N, ▽ 10.A; dashed lines, ◊ 40.C, Δ 81.H, X 10.B, ▽ 97.C.

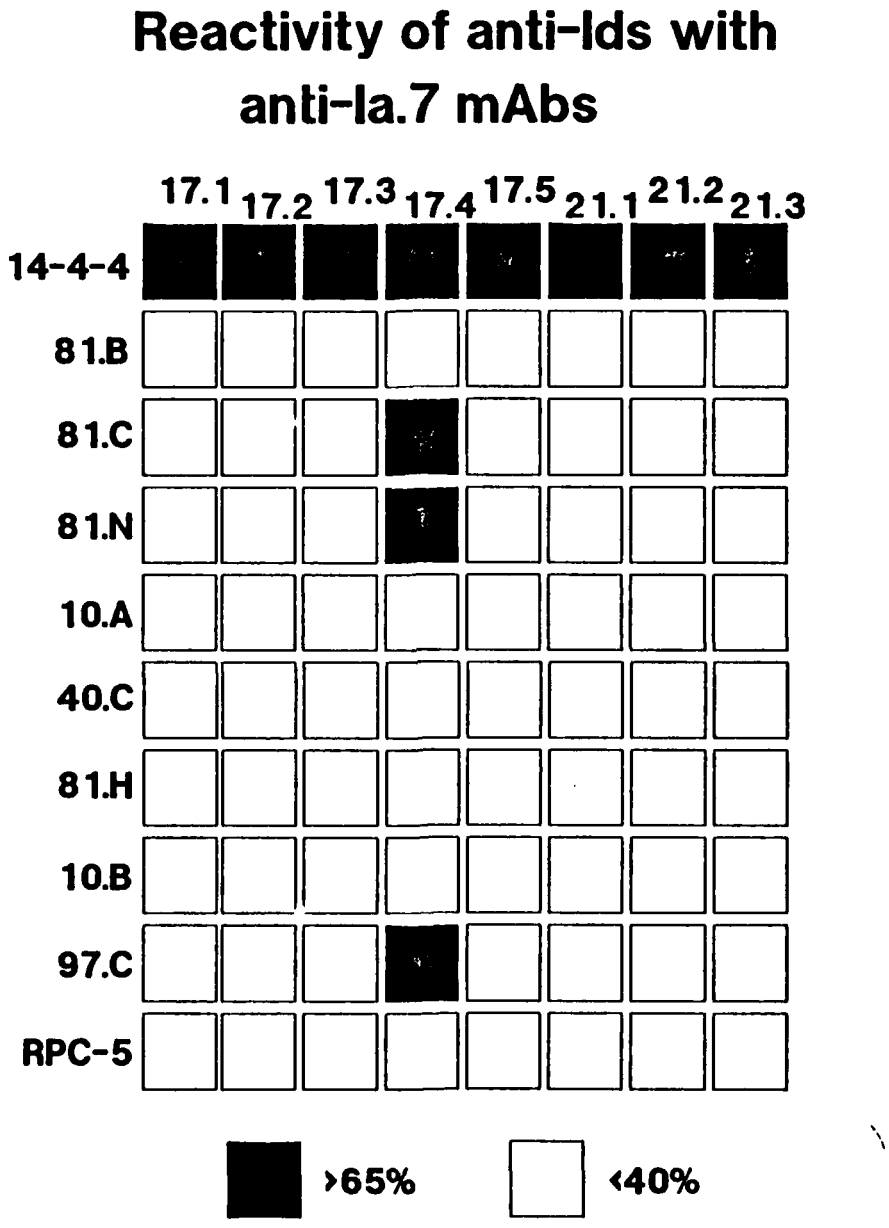


Fig. 16. Idiotypic cross-reactions of anti-Ia.7 hybridomas. Inhibition ELISA assays were performed as described in Materials and Methods. All inhibitors were purified and were titrated in the experiment. Data are presented for the top concentration tested which was 50 μg/ml.

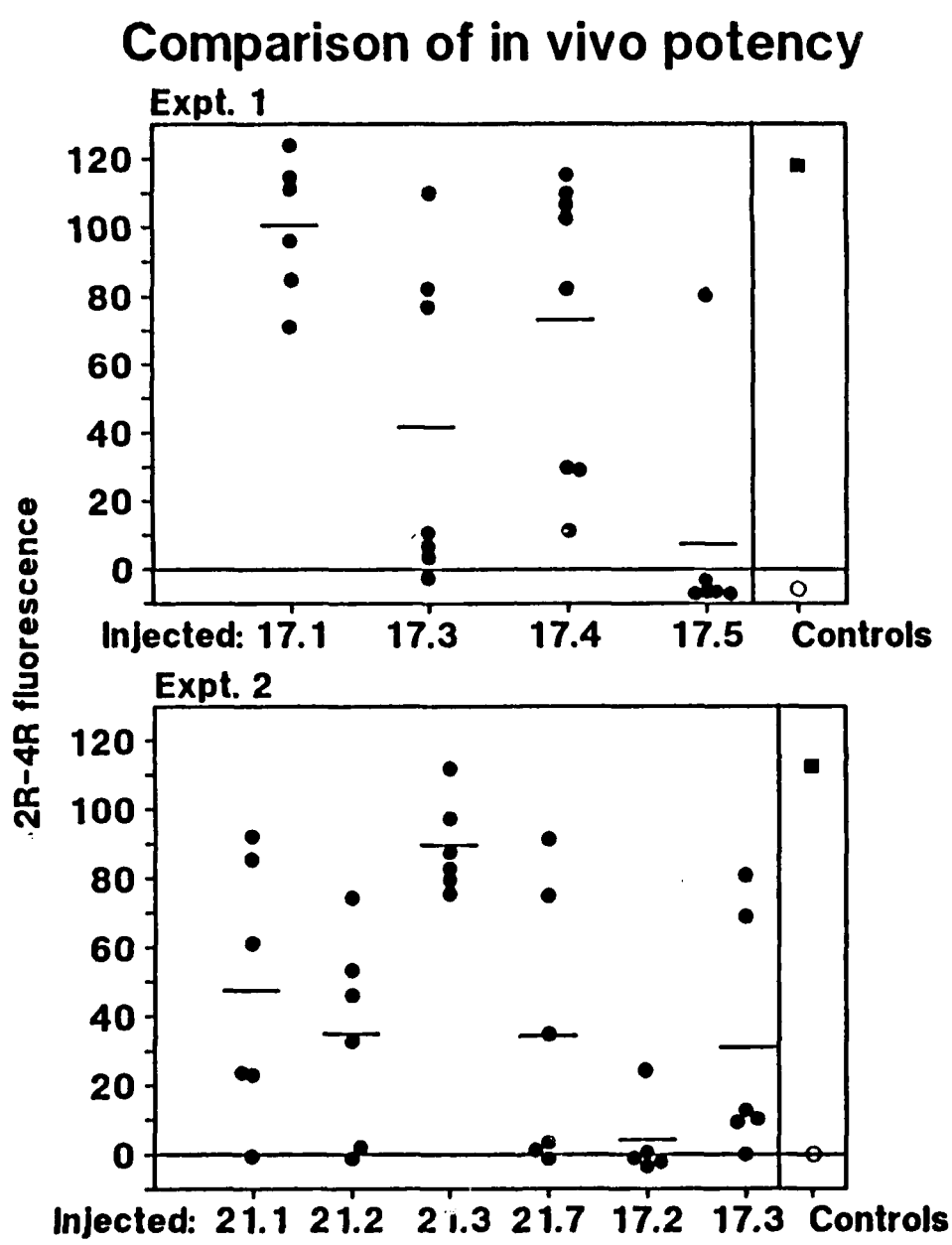


Fig. 17. Comparison of in vivo potency of monoclonal anti-idiotopes in inducing anti-I-E responses. Mice were immunized with mAnti-Id-KLH, two 20 μg doses as described in Materials and Methods. I-E specific activity was measured by flow cytometry as the difference in mean channel fluorescence between 2R and 4R cells. Staining is for IgG1 in the case of test sera and for IgG2a in the case of 14-4-4 as a control. Controls: ■ 14-4-4; ○ normal mouse serum. For the groups, each dot represents an individual mouse, and the horizontal lines represent group mean values.

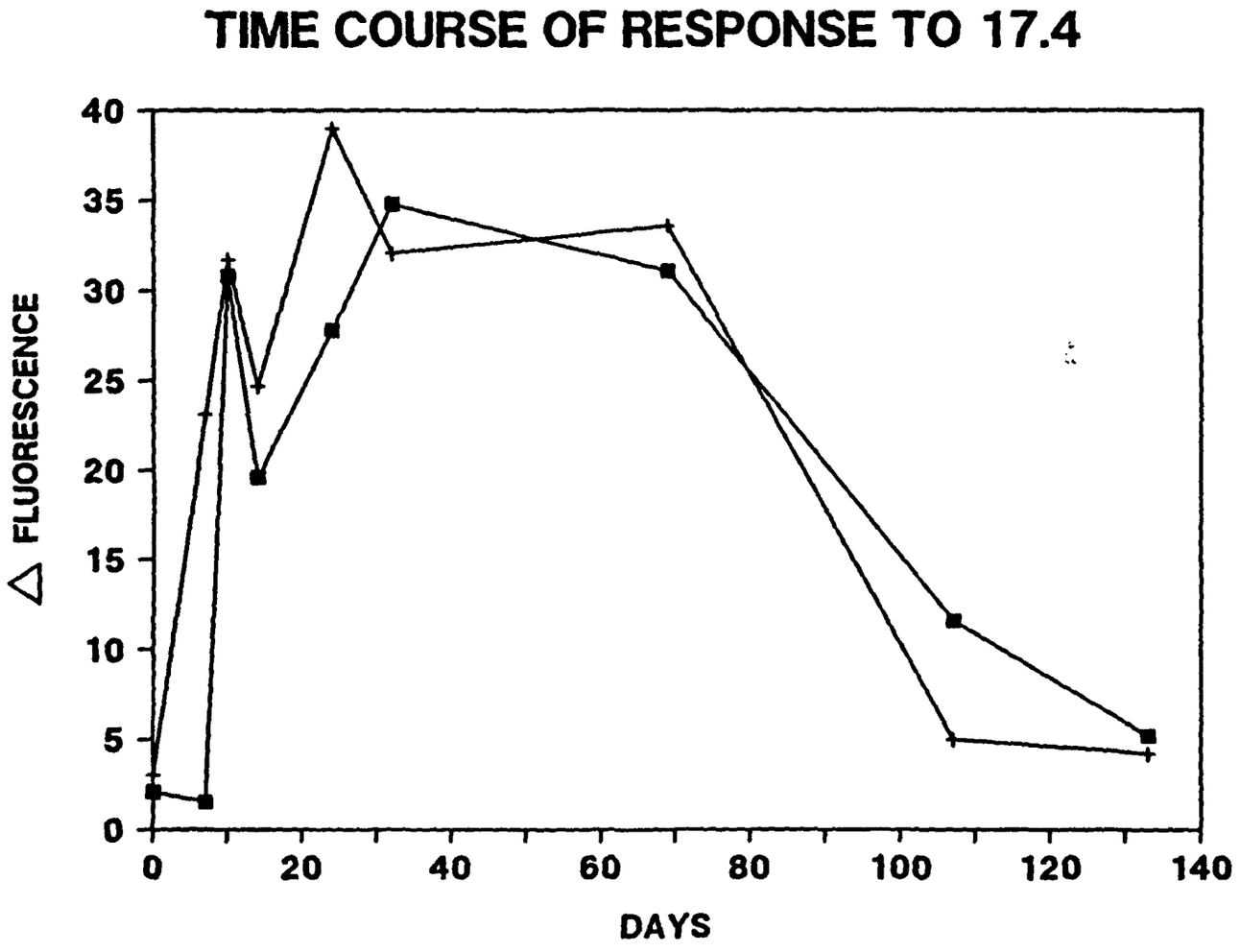


Fig. 18. Time course of anti-I-E response in mice immunized with 17.4-KLH. Flow cytometry performed as in Fig. 13. The two curves represent responses in two individual mice.

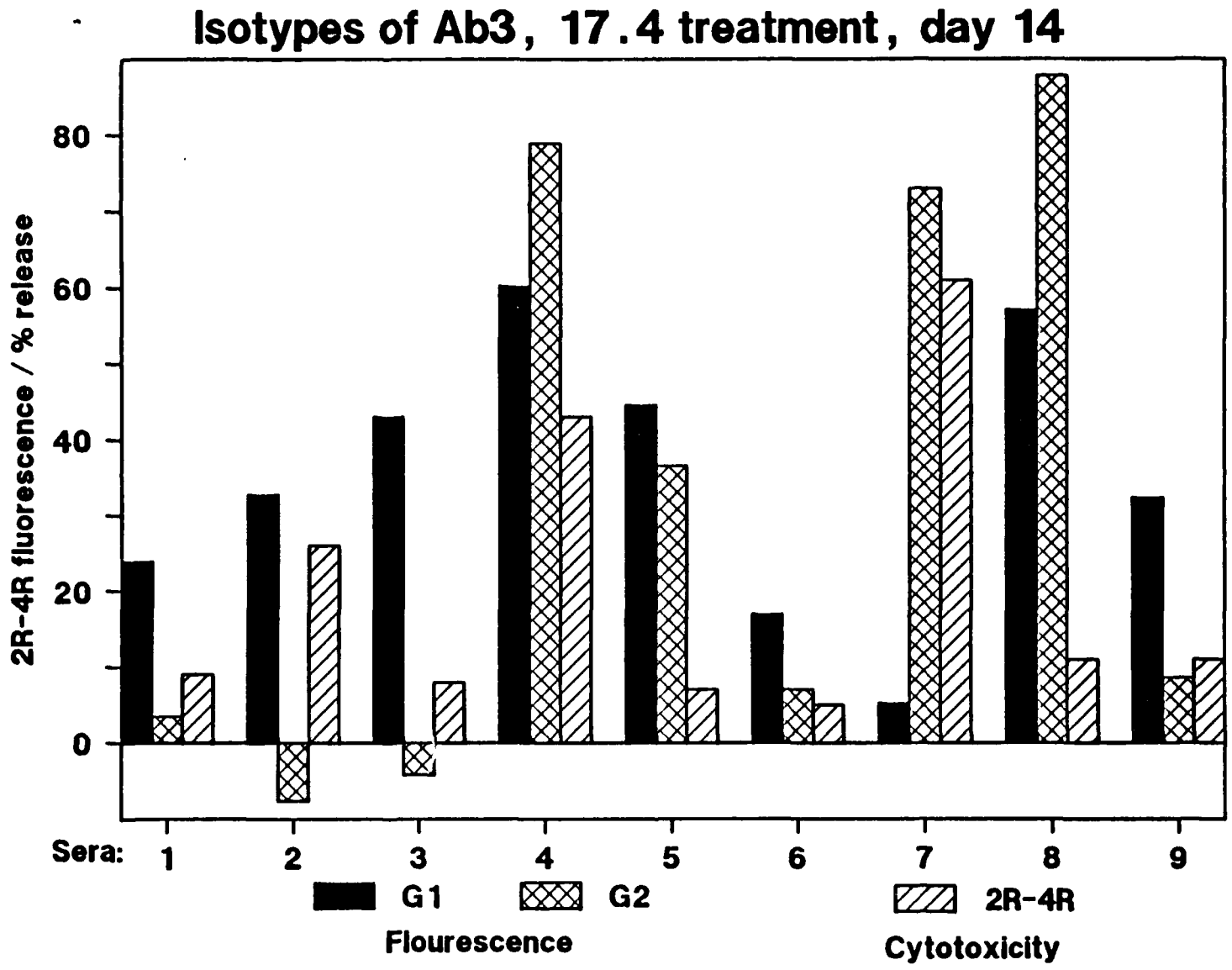


Fig. 19. Ig isotypes in the response induced by anti-idiotope treatment. IgG1 and IgG2a levels specific for I-E were measured by flow cytometry as in Fig. 13. Cytotoxic assays were performed on B10.A(2R) targets as an indication of possible IgM activity.

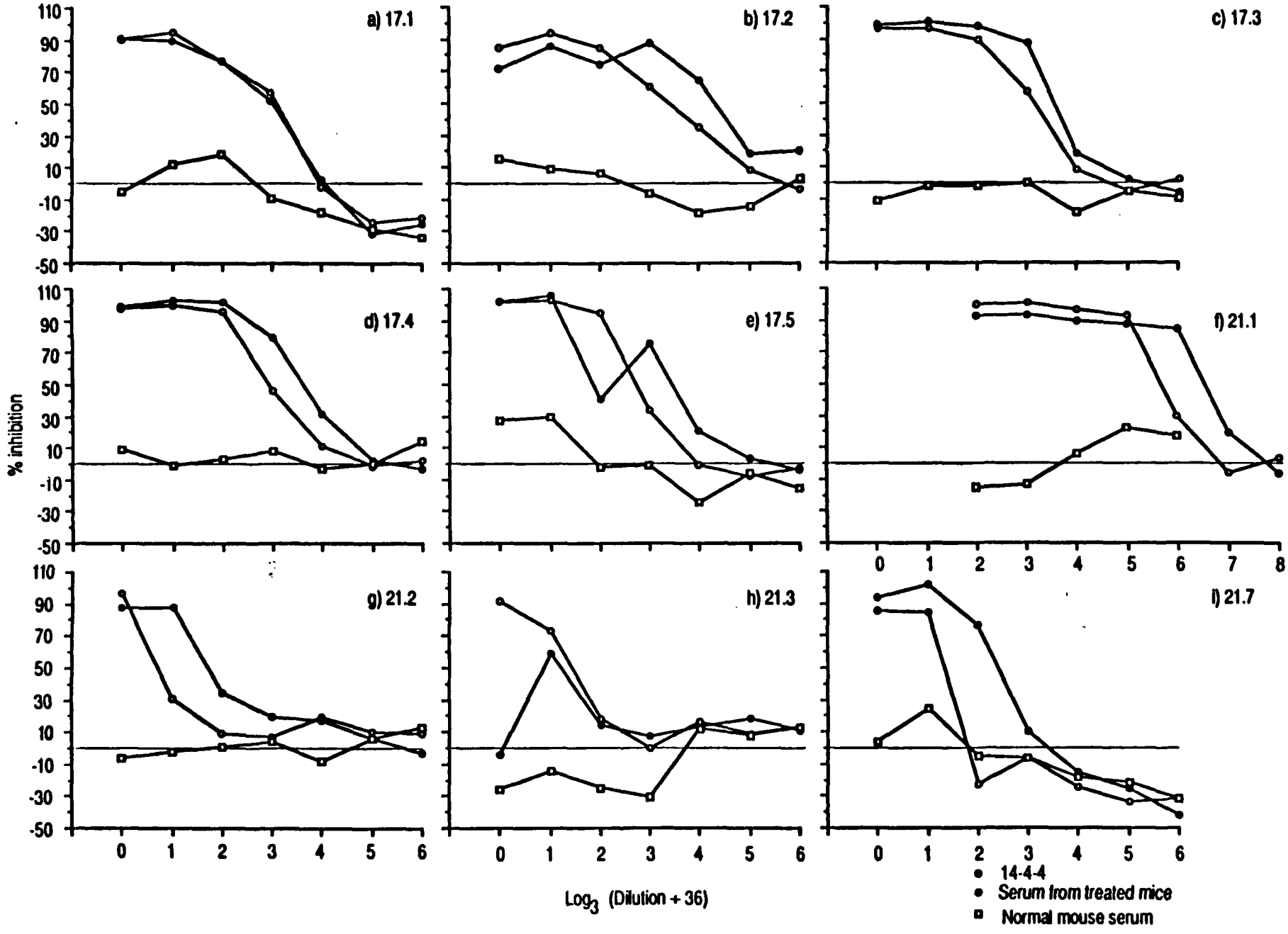


Fig. 20. Ab3 induced by *in vivo* treatments. Sera of immunized mice were tested by ELISA for ability to inhibit the binding of homologous mAnti-Id to 14-4-4. Sera were pooled from the same groups tested in Fig. 13. Inhibitors: ○ 14-4-4, stock concentration 0.471 mg/ml; ● serum from mAnti-Id treated mice; □ pooled C3H.SW normal serum.

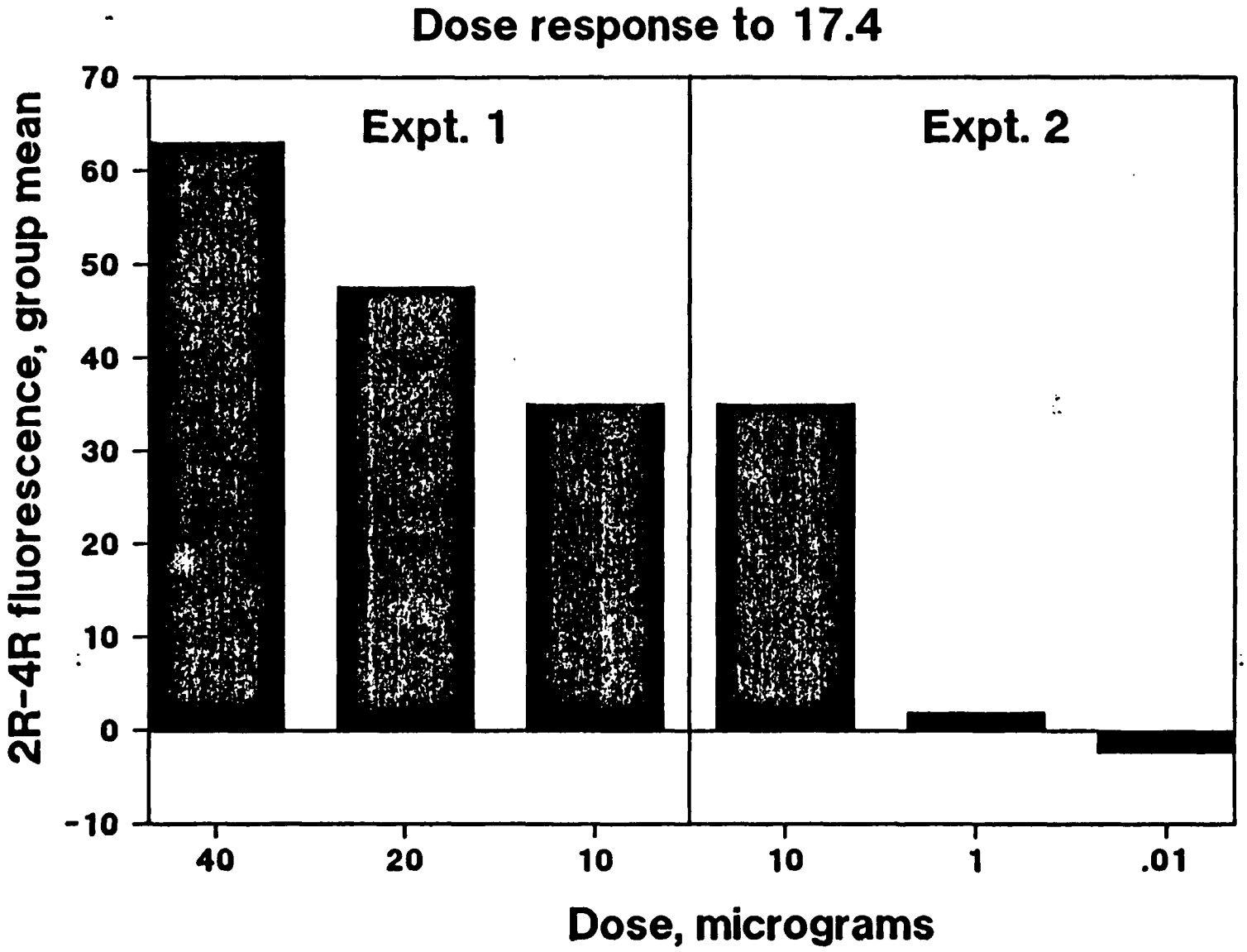


Fig. 21. Dose response for immunizations with 17.4. Mice were immunized once with 17.4-KLH in CFA ip, and tested at 6 weeks for I-E-specific IgG1. Doses refer to the mAnti-Id content. Sera were tested individually, and mean fluorescence values then averaged.

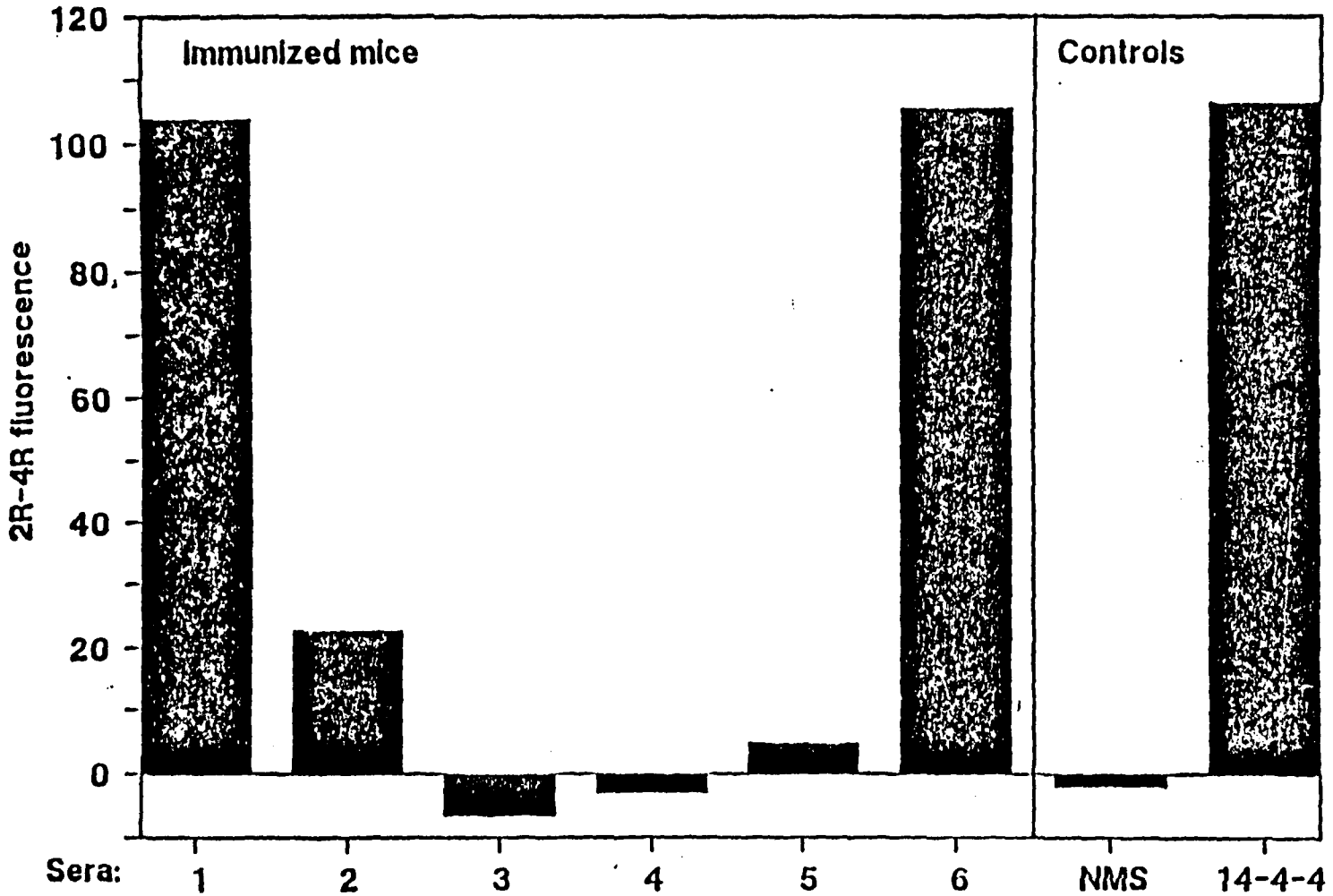


Fig. 22. *In vivo* response to high dose of 17.5. Mice were immunized with two doses of 17.5-KLH (100 μg mAnti-Id each). Immune and normal serum controls were detected with anti-IgG1 staining. 14-4-4 was detected with anti-IgG2a staining.

COMBINING SITE STRUCTURE

OF INDUCED ANTI-I-E

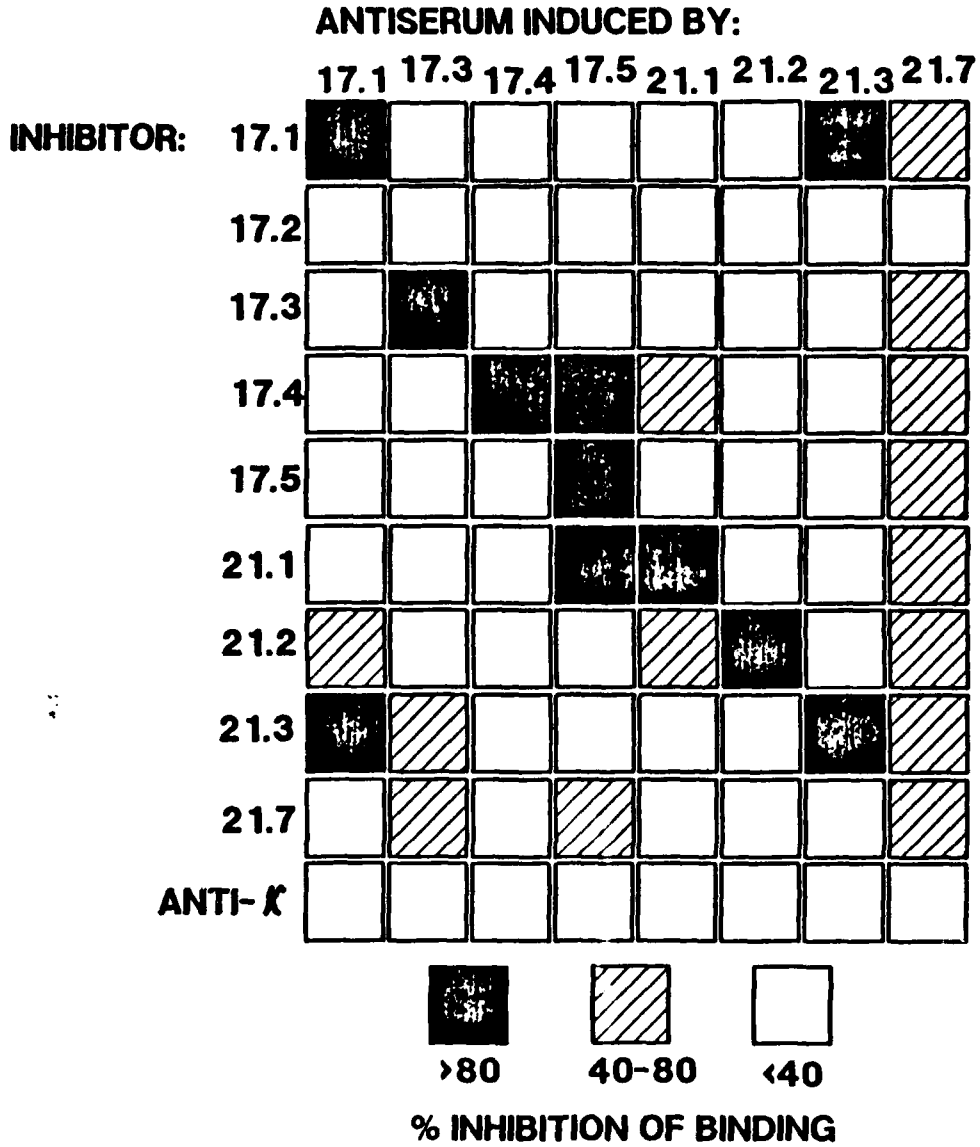


Fig. 23. Combining site structure of antibody popoulations induced by anti-Ids. Mice were immunized by the mAnti-Ids indicated along the top of the matrix, by the usual protocol (as in Fig. 5). Pooled sera from positive responders in each group were used at a dilution determined to be below saturation, so that inhibition would be detectable. Inhibitors indicated along the left side of the matrix were used at 400 μg/ml. Sera were tested on B10.A(2R) and B10.A(4R) cells, and % inhibition refers to % inhibition of I-E specific activity.

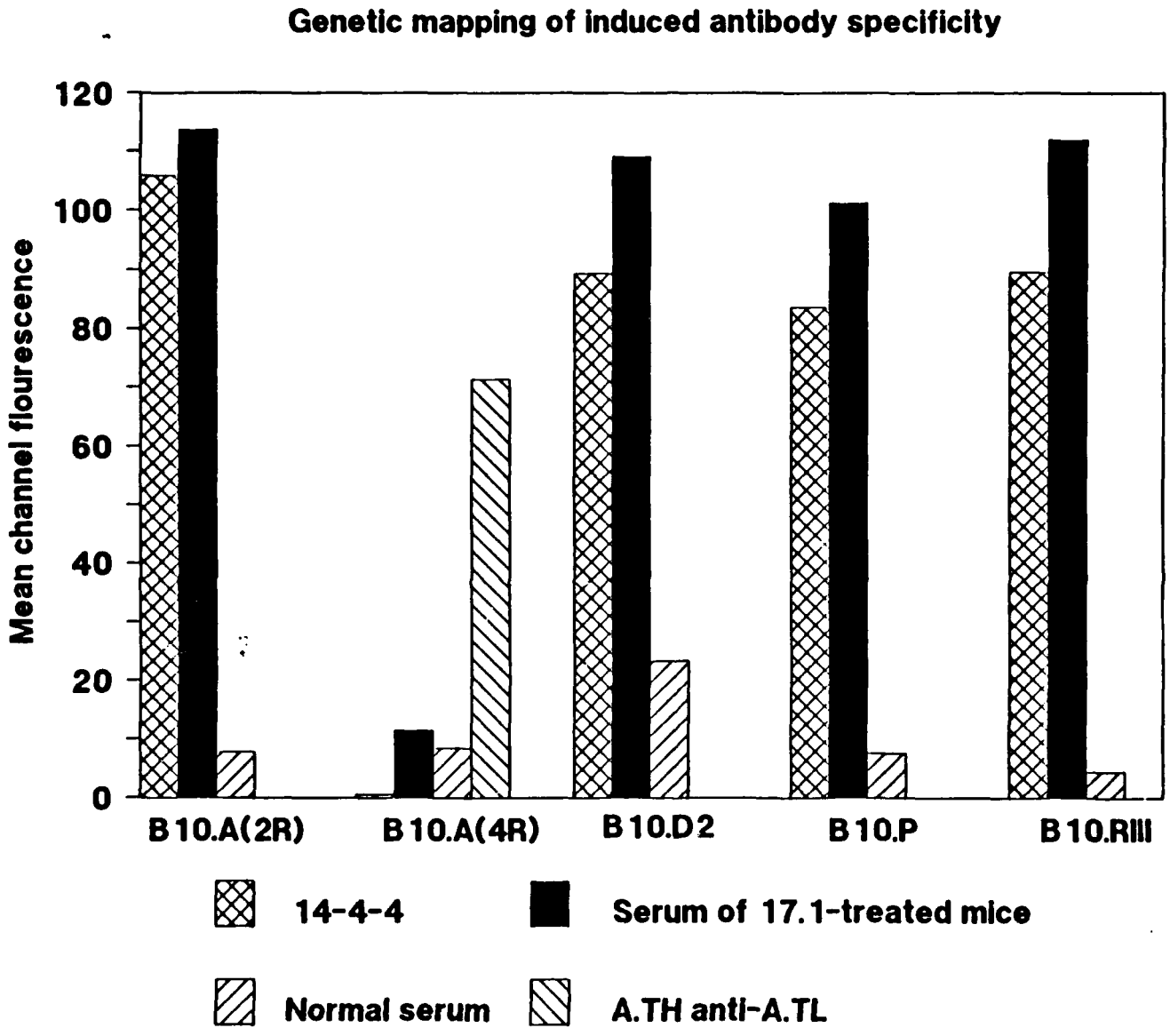


Fig. 24. Genetic mapping of induced antibody specificity. Sera were tested by flow cytometry on LPS blasts from each of the indicated strains. B10.A(2R) and B10.A(4R) map reactivity to the I-E region in the *k* haplotype; the other strains are all of independent haplotypes (B10.D2 is H-2^d, B10.P is H-2^p, and B10.RIII is H-2^r), thus showing that the antibodies react with I-E antigens of four haplotypes, a hallmark of the Ia.7 specificity.