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MOLECULAR RECOGNITION OF ALPHA-NEUROTOXINS

FINAL REPORT

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Peptides representing all the loops and surface regions of α-bungarotoxin were synthesized. Control peptide analogs were also prepared in which these sequences were randomized. The peptides were used to map the profiles of the antibodies and T cells obtained after BgTX immunization. Also, the abilities of anti-peptide antibodies and T cells to recognize the immunizing peptide and BgTX were determined. Immunization of mice with the individual peptides afforded significant protection against BgTX challenge. The most protective peptides, L1, L2 and C-tail, allowed the mice to survive three times the LD_{50} of the controls (i.e., Protective Index = 3). Immunization with an equimolar mixture of the three peptides gave better protection (P.I. = 4.6), while an ovalbumin conjugate carrying these three peptides simultaneously afforded enormous protection (P.I. = 18.1) which was much higher than the protection by BgTX immunization (P.I. = 9.7). It is concluded that the multi-peptide conjugate should serve as an excellent vaccine against BgTX. The work is being prepared for a patent application.

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FOREWORD

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ABBREVIATIONS

AChR, acetylcholine receptor

BgTX, α-bungarotoxin of *Bungarus multicinctus*

BSA, bovine serum albumin

CFA, complete Freund's adjuvant

LNC, lymph node cells

PBS, 0.15 M NaCl in 0.01 sodium phosphate buffer, pH 7.2

PI, protection index, the ratio of the value of LD_{50} of BgTX immunized mice over the LD_{50} of unimmunized mice.

RIA, radioimmune assay

SPECIFICITY OF ANTIBODY AND T-CELL RESPONSES OBTAINED AFTER IMMUNIZATION WITH α-BUNGAROTOXIN OR ITS SYNTHETIC PEPTIDES AND PROTECTION AGAINST TOXIN POISONING BY PEPTIDE IMMUNIZATION

1. INTRODUCTION

The venoms of snakes from the *Elapidae* and Hydrochiidae groups possess a family of compounds which have very pronounced pharmacological activities (Dufton and Hider, 1983; Endo and Tamiya, 1986). These include long (between 65 and 74 residues) and short (between 60 and 62 residues) neurotoxins known to bind specifically and tightly to the α-subunit of the nicotinic acetylcholine receptor (AChR) (Mennier *et al.*, 1974; Webber and Changeux, 1974; Lee, 1979; Haggerty and Froehner, 1981). AChR plays a central role in postsynaptic neuromuscular transmission by mediating ion flux across the cell membrane in response to binding of acetylcholine (Karlin, 1980; Conti-Tronconi and Raftery, 1982; McCarthy *et al.*, 1986; Changeux *et al.*, 1984; Hucho, 1986). Binding of neurotoxin to AChR is very tight (K_d in the range of 10^{-11}M) leading to relatively permanent closure of the ion channel and blockage of the action of acetylcholine. α-Bungarotoxin (BgTX) is a long (74 residues) neurotoxin found in the venom of *Bungarus multicinctus*. The binding sites for AChR on BgTX were recently mapped by synthetic peptides representing each of the BgTX loops (McDaniel *et al.*, 1987; Atassi *et al.*, 1988). Conversely, the toxin-binding sites on the α-subunit of the *Torpedo* (Mulac-Jericevic and Atassi, 1986, 1987a,b) and human (Mulac-Jericevic *et al.*, 1988; Ruan *et al.*, 1991) AChR were mapped by using synthetic uniform-sized overlapping peptides encompassing the entire extracellular parts of the respective subunit. The region-to-region contacts between BgTX and human AChR were determined by peptide-peptide interactions and molecular modeling of the receptor cavity (Ruan et. al.,

1990).

In the present work, the synthetic BgTX loops were examined for their ability to bind antibodies and stimulate T-lymphocytes obtained after BgTX immunization. Conversely, the abilities of antibodies and T cells, obtained after immunization with various BgTX peptides to recognize the parent BgTX were determined. The purpose of this immunological mapping was to identify the immunodominant BgTX regions which were then employed as immunogens to confer protection against toxin poisoning.

2. Experimental Procedure

2.1. Materials

α-Bungarotoxin from the venom of *Bungarus multicinctus* was obtained from Miami Serpentarium Laboratories (Punta Gora, Florida). The purity of the toxin was confirmed by high pressure liquid chromatography. Synthesis, purification and characterization of the peptides corresponding to the various loops and exterior regions of BgTX have been reported in detail (Atassi *et al.*, 1988). In addition, we have prepared for the present work three peptides corresponding to: R.L1/N-tail, randomized sequence of the loop region 1-16 or BgTX; R.L2, randomized sequence of the loop region 26-41 of BgTX; and R.L3/Ext, randomized sequence of the loop region 45-59 of BgTX. These peptides were synthesized, purified and characterized as described (Atassi *et al.*, 1988). The BgTX peptides and their randomized counterparts are shown in Fig. 1. Other peptides, that are unrelated to BgTX, were obtained from our extensive library of synthetic peptides.

2.2. Antisera

Antisera against BgTX were raised in rabbits and mice. For preparation of rabbit antisera, 3-months old (5-7 lbs) New Zealand white rabbits (Ray Nichols, Lumberington, TX) were immunized subcutaneously at several sites with an emulsion (200 μl) of equal volumes of complete Freund's adjuvant (CFA) and a solution of BgTX (10 μg) in 0.01M sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS). The rabbits were injected with booster injections (of a similar BgTX dose, except that the boosters were given in incomplete Freund's adjuvant) three weeks after the first injection and thereafter monthly. The antisera used in this study were obtained 62 days after the first immunization.

Mouse anti-BgTX antisera were prepared in the following mouse stains: outbred (ICR), C57/BL6 (H-2^b), SJL (H-2^s) and Balb/c (H-2^d). The mice were purchased from the National Cancer Institute, and Jackson Laboratory (Bar Harbor, ME). The mice were each immunized subcutaneously at multiple sites with an emulsion (50 μl) of equal volumes of CFA and a solution of BgTX (4 μg) in PBS. They received three booster injections of a similar dose (using incomplete Freund's adjuvant in the boosters) two weeks apart and thereafter they were boosted and test bleed every three weeks. Antisera used in these studies were obtained 56 days after the initial immunization.

2.3. Radioimmunoadsorbent titrations of anti-BgTX antisera

BgTX, its peptides and control proteins and peptides were conjugated to Sepharose CL-4B as previously described (Twining and Atassi, 1979). Quantitative radioimmunoadsorbent titrations were performed in PBS containing 0.1% BSA. A fixed amount of ^{125}I-labeled anti-BgTX antibody (1x10^5 cpm) was reacted with increasing amounts of adsorbent suspension (1:1 v/v in PBS-0.1% BSA), at 4^{o}C for 16 hrs with gentle rocking, in a reaction volume of 260 μl. After reaction, the adsorbents were washed on the centrifuge 5 times with PBS, transferred quantitatively to clean tubes and then counted on a gamma counter.

2.4. Lymphocyte proliferative assay

To determine the T-cell recognition regions o.. BgTX, mice were immunized subcutaneously at the base of the tail with 4 μg of BgTX as an emulsion (100 μl) of equal volumes of the BgTX solution in PBS and CFA. Seven days after priming the inguinal and

periaortic lymph nodes were aseptically removed and a single cell suspension was prepared for proliferative assay. The regions that are recognized by BgTX-primed LNC were mapped in 5 mouse strain: SJL (H-2^s), C57BL/6 (H-2^b), C3H/HeNCr (H-2^k), CBA/JNCr (H-2^k), Balb/c AnNCr (H-2^d).

To prepare peptide-primed lymphocytes, the mice were immunized with peptide (25 μg/mouse). Otherwise the procedure was the same as that described for the preparation of BgTX-primed LNC. For proliferative assay, single cell suspensions of LNC from primed mice were prepared in Hank's balanced salt solution. The cells were washed and resuspended in RPMI 1640 with 1% normal mouse serum and supplemented as described (Bixler *et al.*, 1984). The number of viable cells was determined by vital staining with fluorescein diacetate (Rotman and Papermaster, 1966). Viable LNC ($3x10^5$ cells/well) were cocultured in triplicate with various concentrations of mitogen, BgTX, its synthetic peptides, or control proteins and peptides. Control peptides included synthetic peptides which had the same amino acid composition as the synthetic BgTX peptides, except that their sequence was randomized. After 3 days of incubation at 37°C in a humidified, 5% CO_2 atmosphere, the lymphocytes wee pulsed for 18 hrs with [^{3}H]-thymidine (2 μCi/well) (Research Products International Corporation, Mount Prospect, Illinois) and then harvested onto glass microfiber filters (Whatman, Clinton, New Jersey) for counting by liquid scintillation.

2.5. Preparation of a conjugate carrying three peptides

10 mg of hen ovalbumin (OVA) was dissolved in 2 ml of 0.15 MNaCl solution and then 2 mg of each peptide (L1, L2, C-tail) was added. After mixing for 30 minutes, 3

molarexcess of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was added. The reaction mixture was stirred overnight at room temperature after which the conjugate was dialyzed against distilled water and lyophilized. The extent of coupling (i.e. moles of each peptide coupled per mole of OVA) was determined by amino acid analysis of acid hydrolysates (constant boiling HCl, sealed under nitrogen, 110°C, 24, 48 and 72 hr) of the conjugate. This showed that the conjugate contained 2.12 moles of L1, 11.34 moles of L2 nd 8.85 moles of C-tail per mole of OVA ($L1_2L2_{11}$C-$tail_9$-OVA).

2.6. Immunization procedure to determine the protective capacity of the individual free peptides

Each peptide was injected, in its free form (i.e. without coupling to any carrier), into 45 mice each of Balb/c ($H\text{-}2^d$) and SJL ($H\text{-}2^s$). The peptide (50 μg) was injected into the footpad as an emulsion (50 μl/mouse) of equal volumes of CFA and the peptide solution in PBS. The mice received similar doses of booster injections every 4 weeks and test bleeds were obtained every 3 weeks. The sera monitored by solid-phase plate RIA for their titers of antibodies that bind to the immunizing peptide and to BgTX. When antibody titers appeared to be leveling off, the mice were challenged with BgTX as described below (Section 2.9).

2.7. Immunization procedure to determine protection by peptide mixture

A solution of an equimolar mixture of peptides L1, L2 and C-tail was prepared in PBS (2 mg/ml). Fifty Balb/c mice were each immunized in the footpad with peptide mixture (50 μg) as an emulsion (50 μl) of equal volumes of CFA and the solution of the

equimolar peptide mixture. The mice received similar doses of 8 booster injections. The first three booster injections were given every 2 weeks after which the boosters were given every 3 weeks. Monthly test bleeds were obtained and monitored for the titers of antibodies that will bind to the immunizing mixture and to BgTX. The sera from the individual mice were not mixed but were studied separately. When antibody titers appeared to be leveling off, the mice were challenged with BgTX as described below (Section 2.9).

2.8. Immunization procedure to determine protection by the three-peptide OVA conjugate

Forty Balb/c mice were immunized with the OVA conjugate which carries three peptides on one molecule ($L1_2L2_{11}$C-tail$_9$-OVA). The antigen (50 μg) was given in the footpad as an emulsion (50 μl) of equal volumes of CFA and the conjugate solution in PBS. The mice were given 8 booster injections of similar doses. The first three injections were given every 2 weeks and the last five were given every 3 weeks. Test bleeds were obtained from each mouse monthly and were studied separately (i.e. they were not mixed) for their titers of antibodies that will bind to BgTX. When antibody titers had leveled off, the mice were challenged with BgTX as described below (Section 2.9).

2.9. Challenge with BgTX to determine protective immunization by BgTX and its peptides

Different doses of BgTX, as solutions (40 μl) in PBS, were injected i.v. (in the tail). The number of mice surviving BgTX challenge was plotted against the respective challenge doses. The BgTX challenge dose at which 50% of the mice survived was termed the LD_{50}

value. The ratio of the LD_{50} value after immunization with a given antigen (BgTX, BgTX peptides, random sequence control analogs and other proteins and peptides) to the LD_{50} of unimmunized mice is termed Protection Index (PI).

3. RESULTS

3.1. Profiles of the antibody and T-cell responses obtained after BgTX immunization.

3.1.1. Binding of rabbit and mouse (outbred) anti-BgTX antibodies to BgTX and synthetic peptides. Radioimmunoadsorbent titrations were performed with rabbit and mouse ^{125}I-labeled anti-BgTX antibodies. The results of reaction of the peptides with rabbit anti-BgTX are summarized in Fig. 2. Peptides C-tail, L1,L1/N-tail, L2 and L4/C-tail showed strong antibody binding, decreasing in that order. The remaining peptides (L3/Ext. L2G and L3) bound low amounts of antibody. Randomization of the amino acid sequences of peptides L1/N-tail, L2 and L3/Ext gave peptides (i.e. R.L1/N-tail, R.L2 and R.L3/Ext., respectively) which were unable to bind anti-BgTX antibodies (see Fig. 2). Furthermore, anti-BgTX antibodies did not bind to adsorbents of unrelated proteins and peptides. The titer of the rabbit antiserum was determined using a double antibody assay and 50 μl of adsorbent suspensions (1:1, vol/vol) in PBS/0.1% BSA. Binding to BgTX and to its peptides was determined at dilutions of rabbit anti-BgTX from 1:500 up to 1:5000 (vol/vol, in PBS/0.1% BSA). The results (Fig. 3) showed that even at dilutions of 1:5000 considerable amounts of antibodies could be bound by the peptides C-tail, L1, L1/N-tail and L2. We have determined the binding of three mouse anti-BgTX antisera with the eight synthetic toxin peptides. Figure 4 gives an example of quantitative radioimmunoadsorbent titration of an ^{125}I-labeled mouse anti-BgTX (mouse #236) with the various toxin peptides

and controls. Table 1 summarizes the maximum (plateau) binding values of the toxin peptides and controls. With mouse anti-BgTX antibodies, the following peptides exhibited antibody reactivity in decreasing order: L1, L1/N-tail, C-tail, L4/C-tail and L2 (Fig. 4). On the other hand, peptides L3, L3/Ext and L2G showed little or no antibody binding which was not significantly different from binding to the randomized peptides and to unrelated proteins and peptides (Fig. 4). From these results, it is concluded that the same BgTX regions are immunodominant regardless of the host species (at least in mouse and rabbit). The strongest antibody binding activities resided in peptides L1, L2 and the C-Tail. Addition of the N-terminal to loop L1 (i.e. peptide L1/N-tail) causes no advantage in terms of bound antibody. Similarly, addition of loop 4 to the C-tail region (i.e. L4/C-tail) does not give any additional binding activity to that expressed by the C-tail region alone. Replacement of Trp-28 in L2 by a glycine [L2(G)] causes a large loss in the antibody binding activity of loop 2.

3.1.2. Antibody and T-cell recognition sites of BgTX in toxin-primed independent mouse haplotypes. In order to understand the role of T cell recognition in protection against neurotoxin poisoning, it is important to map, in selected mouse strains, the regions that are recognized by T cells. Comparison of these to the regions recognized by antibodies in the *same* mouse strains should serve to identify the toxin regions that would be most efficient in active immunization for protection against toxin poisoning. Five mouse strains (SJL,H-2^s; C57/BL6, H-2^b; C3H/HeNCr, H-2^k; CBA/JNCr, H-2^k and Balb/c AnNCr, H-2^d) were studied. The recognition of the toxin peptides by T cells of the five mouse strains are shown in Figures 5-9. The random-sequence analogs of the toxin peptides, myoglobin (Mb), BSA and a nonsense peptide were used as negative controls. The binding of the peptides

to anti-BgTX antibodies raised in these same five mouse strains was determined by quantitative radioimmunoadsorbent titrations of ^{125}I-labeled anti-BgTX antibody with various amounts of peptide adsorbents (from 25 μl to 200 μl of a 1:1, vol/vol suspension). The results of antibody titrations are shown in the attached Figures 10-12. The antibody and T-cell responses in these mouse strains are compared in Table 2. The results show that, at the T cell level, the H-2^b and H-2^d haplotypes are high responders to BgTX while the H-2^s and H-2^k haplotypes are moderate responders.

3.2. Antibody and T-cell responses obtained after peptide immunization

For a peptide to be useful as an immunogen for protection against toxin poisoning it needs to generate an immune response which cross-reacts with the intact toxin. In order to determine their usefulness as immunogens, the synthetic BgTX peptides were immunized individually into two mouse strains (Balb/c and SJL). The abilities of the antibodies and T cells obtained after peptide priming to recognize the intact toxin were determined.

3.2.1. Reaction of anti-peptide antibodies with BgTX and with the immunizing peptide. Each of the synthetic BgTX peptides was immunized into 8-10 mice each of Balb/c and SJL. The antisera against each peptide in individual mice were not mixed but were studied independently. The results of binding of anti-peptide antibodies to the immunizing peptide and to BgTX are summarized in Tables 3 and 4. With both Balb/c and SJL, each of the peptides gave in each mouse antisera that bound to the immunizing peptide. However, not all the antisera against a given peptide were able to bind to whole BgTX. The number of mice that gave antibodies which bound to whole BgTX varied with the immunizing peptide. In both mouse strains, anti-peptide antibodies that recognized intact BgTX were obtained

in a higher proportion of mice after immunization with L2 than with any of the other peptides (Tables 3 and 4). However, the differences among the groups of mice that gave toxin-binding anti-peptide antibodies were not dramatic. It was therefore decided to test each of the BgTX synthetic peptides as an immunogen for its ability to generate protective antibodies.

3.2.2. Proliferative response of peptide-primed T cells to the immunizing peptide and to BgTX. Using the synthetic BgTX peptides as immunogens, we have determined the ability of T cells obtained from mice that had been primed with a given peptide to proliferate *in vitro* to the peptide and to the whole BgTX molecule. The experiments were carried out in two mouse strains, Balb/c (H-2^d) and SJL (H-2^s). A fixed number ($5x10^5$ cells/well) of LNC from mice that had been immunized with an optimum dose (25 μg/mouse) of each peptide were challenged *in vivo* with different doses of the peptide or whole BgTX. In addition, we used as a control a synthetic peptide containing the same amino acids as the immunizing BgTX peptide except that their sequence was randomized. Typical dose response curves are shown in Figures 13 and 14. Figure 13 gives an example (peptide L3/Ext) for Balb/c T cells while Fig. 14 shows an example (L4/C-tail) for T cells of SJL. Tables 5 and 6 summarize the maximum proliferative responses, mounted in response to peptide challenge, by peptide-primed T cells of Balb/c and SJL mice, respectively. The results in the tables were not corrected for the amount of label incorporated by the controls. The corrected results are shown schematically in Figures 15 and 16 for Balb/c and SJL, respectively. The experiments indicate that Balb/c responds strongly to all the BgTX peptides, except peptide L1. However, the ability of Balb/c peptide-primed T cells to recognize BgTX varied. Thus, whereas peptide C-tail evoked the highest response of any peptide to itself, these T cells

showed little or no recognition of intact toxin. The peptide-primed T cells of Balb/c recognized intact BgTX in the following decreasing order (Fig. 15): L3/Ext, L2=L4/C-tail, L3, L1, C-tail. In SJL, the order of BgTX recognition by peptide-primed T cells was, in decreasing order (Fig. 16): L4/C-tail, L1, C-tail, L2, L1/N-tail. The other peptides evoked little or no T-cell responses in this strain. The results clearly indicated that the T-cell responses to BgTX peptides are under Ir gene control.

3.3. Protection against BgTX by immunization with the single free peptides

In order to investigate the protective ability of the peptides, each peptide was injected in its free form (i.e. without coupling to any carrier) into 45 mice each of Balb/c and SJL strains. The mice received five booster injections with the respective peptide, at which time (12 weeks) they had developed high titers of anti-peptide antibodies in their antisera, as determined by solid-phase RIA. The mice were challenged with different doses of intravenous (in the tail) injections of BgTX. Each challenge dose was administered to 5 mice. The number of mice surviving BgTX challenge was plotted as a function of the BgTX challenge dose. For controls, the randomized peptides (Fig. 1) were each injected into 45 mice and challenged with intact toxin in exactly the same way as was done for the mice immunized with the BgTX peptides. Additional controls included unimmunized mice (45) and mice (45) that were immunized with intact BgTX. The protection results for Balb/c are summarized in Figures 17 and 18 while those for SJL are shown in Figures 19 and 20. The protection parameters for both strains with each of the peptides are summarized in Table 7. It can been seen that, in both Balb/c and SJL, each of the peptides afforded significant protection against BgTX challenge (PI = 2.2-3.2 relative to

control mice). The highest protection was afforded by peptides L2, L1 and C-tail (LD_{50}, 3.2 times higher in Balb/c and 2.7 to 2.5 times higher in SJL than the respective control mice, see Table 7). None of the peptides displayed the protection levels obtained by BgTX immunization (PI: Balb/c, 9.7; SJL, 7.4).

3.4. Design of multi-peptide vaccines

3.4.1. Immunization with an equimolar mixture of the most protective peptides. In view of the finding that, in both Balb/c and SJL strain mice, peptides L1, L2 and C-tail, when each was used singly as an immunogen, generated immune responses that were most protective against BgTX poisoning, we investigated whether protection would be enhanced (i.e. the mice would survive a higher BgTX challenge dose) if all three peptides were used together as an immunogen. An equimolar mixture of peptides L1, L2 and C-tail was injected into 50 Balb/c mice and the mice were boosted 8 times with the same mixture. Antisera were obtained from these mice prior to challenge with BgTX in order to determine in each mouse the level of antibodies that will bind to intact BgTX. The mice were then challenged with different doses of BgTX. Figure 21 correlates the results of protection with the level of antibodies that bind to whole BgTX. The results showed that the mixture of the peptides L1, L2 and C-tail afforded better protection (LD_{50}, 14.63 μg) than any one of the peptides by itself. Survival to a given challenge dose was somewhat related to the level of antibodies that will bind to whole BgTX (Figure 21).

3.4.2. Immunization with a multi-peptide conjugate. The three most protective peptides L1, L2 and C-tail were coupled to a single carrier, ovalbumin. The three-peptide conjugate was immunized into Balb/c mice which were boosted (8 times) until they mounted high titers of antibodies that bound to whole BgTX. The mice were challenged with various doses of BgTX. Figure 21 gives the relationship between the outcome of challenge with different doses of BgTX and the level of antibodies that bind to BgTX. The results revealed a good relationship between survival to a BgTX challenge dose and antibody binding to BgTX. Mice with high titers survived challenge doses as high as 58 μg BgTX (PI = 18.1). Thus, the three-peptide conjugate afforded protection was almost double that obtained with BgTX (PI = 9.69, see Table 7).

4. Discussion

To design the most efficient peptide vaccine against BgTX poisoning we decided to approach the question in a systematic manner. In order for a peptide to be protective against BgTX poisoning, the peptide should represent an immunodominant region on BgTX and when the peptide is used as an immunogen it should stimulate immune responses that are able to recognize the intact toxin. This is obligatory if the anti-peptide responses are expected to display any neutralizing activity against BgTX. Both antibody and T-cell responses were studied.

When intact BgTX was used as an antigen in rabbits or outbred mice, the strongest antibody-binding activities were directed against regions residing in peptides L1, L2 and C-tail. The same regions were immunodominant regardless of the host species (at least in mouse and rabbits). This is consistent with what is known about antibody recognition of

proteins in outbred animals (Atassi, 1975, 1978, 1984). In independent mouse haplotypes, on the other hand, the immunodominance of various BgTX regions varied with the haplotype which is indicative of genetic control operating at the antigenic site level. It is well established that, in the immune responses to a multi-determinant complex protein antigen, the responses to each determinant (both antibody and T cell) are under separate genetic control (Okuda *et al.*, 1979; Twining *et al.*, 1981; David and Atassi, 1982). In a given mouse strain, the regions on a protein antigen that are recognized by antibodies and by T cells may coincide but there might also be regions on the protein that are recognized by antibodies and for which no detectable T-cell responses are found and/or conversely T-cell recognition regions for which no antibodies are detectable (Bixler and Atassi, 1983, 1984a,b, 1985; Atassi, 1984; Bixler *et al.*, 1984). The results with the antibody and T-cell recognition of BgTX are consistent with these observations.

When the peptides were used as immunogens, antibodies against L2 showed the highest binding ability with intact BgTX. Antibodies against the remaining peptides did not show any significant differences in their binding to BgTX. It was, therefore, decided to examine the anti-peptide T-cell responses. In a given mouse strain, the T-cell response obtained after peptide immunization did not necessarily correlate with whether the immunizing peptide represented an immunodominant T-cell epitope on BgTX (i.e. when BgTX is used as the immunizi ; antigen). The results also indicate that the T-cell responses to the BgTX peptides (when the free peptides are used as immunogens) are under Ir gene control. Since the differences in the abilities of the antibodies against the various peptides to bind intact BgTX were not very significant and since these activities did not necessarily correlate with the ability of anti-peptide T cells to recognize BgTX, it was decided to test each of the peptides for its capacity to generate protective immune

responses.

In both Balb/c and SJL, peptides L1, L2 and C-tail were most protective against BgTX poisoning (PI: Balb/c, 3.2; SJL, 2.5-2.7). Protective immunity exhibited by the other peptides was also quite substantial (PI: Balb/c, 2.5-2.6; SJL, 2.2-2.4). It is noteworthy that the three most protective peptides (L1, L2 and C-tail) were also immunodominant in terms of binding of anti-toxin antibodies, suggesting perhaps that, for identification of the most protective regions, it would have been sufficient to map the immunodominant regions towards anti-BgTX antibodies.

Since each of the peptides L1, L2 and C-tail was quite protective (increasing the D_{50} of BgTX about 3 fold relative to control mice), it was important to determine whether higher protection will be achieved by immunizing mice with all three peptides simultaneously. These studies (which were done only in Balb/c) clearly showed that this was indeed the case. Immunization with an equimolar mixture of the peptides allowed the mice to survive BgTX challenge doses which were 4.6 fold higher than control mice. In other words, immunization with an equimolar mixture of peptides L1, L2 and C-tail was 42% more protective, in terms of survivable BgTX challenge dose, than any of the three peptides by itself. Clearly, antibodies against all three regions are more efficient at neutralizing toxin poisoning than antibodies against any single region. The protective capacity of the peptide mixture was somewhat related to the titer of the fraction, in anti-peptide antibodies, that binds to BgTX. But the titers of these antibodies were moderate and did not increase substantially over an extended period of immunization. It was therefore decided to determine the protective ability of a peptide-carrier conjugate.

The three peptides L1, L2 and C-tail were conjugated to a single carrier. Analysis of the conjugate showed that the coupling levels of the peptides differed. This is to be

expected because each peptide has different reactivity of side chains and accessibility requirements on the surface of the OVA carrier. It was important to find that the conjugate generated high titer antibodies that bound to intact BgTX. This immunogen (i.e. the conjugate) afforded excellent protection against BgTX challenge (PI = 18.1). In fact, the multi-peptide conjugate was almost twice as protective as whole toxin immunization (PI = 9.7). In addition, unlike BgTX, the multi-peptide conjugate is not toxic and, therefore, there is no risk of poisoning the recipient by the immunogen in the process of vaccination. Clearly, the multi-peptide conjugate will constitute an excellent vaccine against toxin poisoning. Thus the prime goal of this research contract, which was to design peptide vaccines against BgTX, has been achieved. These results have been approved by Baylor College of Medicine Patent Committee for patent application. The patent is being prepared by the patent attorneys (see attached letter from Arnold, White and Durkee).

5. CONCLUSIONS

We have mapped, by synthetic peptides, the antigenic regions of BgTX that are recognized by rabbit and mouse anti-BgTX antibodies. Three regions residing within peptides L1, L2 and C-tail were immunodominant. The regions recognized by BgTX-primed T cell were also mapped in five mouse strains. Immunization of Balb/c and SJL mice with each of the synthetic peptides in its free form afforded considerable protection against BgTX poisoning. Peptides L1, L2 and C-tail were most protective and mice immunized with these peptides survived LD_{50} values that were three times higher than control mice. Immunization with an equimolar mixture of the three peptides was even more protective and these mice survived even higher challenge doses of BgTX (4.6 fold

higher than LD_{50} of controls). An OVA conjugate carrying all three peptides, when used as an immunogen, displayed extremely high protection (protection index = 18.1) which was almost double the protection obtained by BgTX immunization (protection index = 9.7). Thus, the main purpose of this contract has been completely achieved. The conjugate of the three peptides should serve as an effective vaccine against BgTX poisoning. The findings have been approved by Baylor College of Medicine Patent Committee for patent application which is now being prepared by the patent attorney for submission to the U.S. Patent and Trademark Office.

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7. PATENTS

This work has been approved by Baylor College of Medicine for a patent application to the U.S. Patent and Trademark Office. Arnold, White and Durkee have been asked by Baylor to prepare the patent for filing.

8. PUBLICATIONS

Because of Baylor's decision to file a patent application, I have been advised by the patent attorney not to publish any of this material prior to the filing of a patent application with the U.S. Patent and Trademark Office (see attached copy of July 17 letter from Arnold, White and Durkee).

The material described in this Final Progress Report will be submitted for publication upon the advice of the patent attorneys. When the work is published, we will submit the required number of copies of reprints to: Commander, US Army Research and Development Command, Attn: SGRD-RMI-S, Fort Detrick, Fredrick, MD 21701-5012.

9. APPENDIX

Letter concerning patent application and publication from Arnold, White & Durkee
Tables
Figures

Table 1. Binding of mouse anti-BgTX antibodies to Synthetic BgTX Peptides

Peptides:	^{125}I-Labeled Antibodies bound (cpm) Mouse # 236	Mouse # 233	Mouse # 235
BgTX	**22015**	**16481**	**17746**
L1	**18715**	**14675**	**15220**
L1/N-tail	16420	13040	14706
L2 (G)	3340	1879	2361
L2	**8590**	**5204**	**6500**
L3	2570	2419	3380
L3/Ext	2340	1796	1830
L4/C-tail	7374	5105	7256
C-tail	**12256**	**16481**	**11588**
Controls			
Random L1/N-tail	962	1132	755
Random L2	1324	875	1092
Random L3/Ext.	1011	962	867
Nonsense	764	103	[illegible]
BSA	1157	895	1121
Myoglobin	1270	725	985

Results were obtained by radioimmunoadsorbent titrations (see Fig. 4,) and represent the average plateau values of three replicate analyses which varied ± 1.3% or less.

Table 2. Comparison of the specificities of antibody and T-cell responses against BgTX in SJL ($H\text{-}2^s$) and C57/BL6 ($H\text{-}2^b$) mice.

	SJL		C57/BL6		Balb/c	
	Antibody	T Cell	Antibody	T Cell	Antibody	T Cell
L1	+ + + +	+	+ + +	+	+ + + +	+ + + +
L1/N-tail	+ + +	+	+ +	+	+ + +	- +
L2	+ +	+ +	+	+	+	+ + +
L3	-	-	-	-	+	+ +
L3/Ext	+	+	-	+	+	+ +
L4/C-tail	+ +	+ +	+ +	+ +	+ +	+ + + +
C-tail	+ + + +	+ +	+ + +	+ +	+ + + +	+ + + +
BgTX	+ + + + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +

Table 3. Binding of Moā αBgTX and peptides: Balb/c 87-day bleed, 1:500 dilution.

Antigen	Mouse No.	Antibody Binding (net cpm) Peptide	αBgTX	% (and No./total) antisera that bind to BgTX
L1(3-16)	602	61569	94310	40 (4/10)
	603	86127	12937	
	619	67784	10936	
	622	85656	39674	
	621	50971	1880	
	618	43039	1383	
	628	69064	148	
	632	54430	197	
	601	43675	1745	
	612	27101	1111	
L1/N-tail (1-16)				
	643	99888	15835	38 (3/8)
	655	137350	25670	
	664	72179	29257	
	666	139717	1935	
	641	53164	1147	
	642	94038	1030	
	653	63194	136	
	672	106812	672	
L2(26-42)				
	683	68731	10654	56 (5/9)
	687	87378	46038	
	690	112045	14837	
	694	109720	23086	
	699	141346	17961	
	697	75042	328	
	715	128585	106	
	717	45685	1332	
	708	29042	1141	

Table 3 (continued)

Antigen	Mouse No.	Antibody Binding (net cpm) Peptide	αBgTX	% (and No./total) antisera that bind to BgTX
L3(48-59)				
	730	71089	14883	33 (3/9)
	734	53290	18447	
	746	60931	16852	
	759	22921	1602	
	731	44690	312	
	739	51387	316	
	753	54803	1247	
	754	61314	824	
	726	25988	1320	
L3/Ext (45-59)				
	790	29698	557	20 (2/10)
	793	36308	965	
	799	48817	4304	
	800	39226	4487	
	780	20187	984	
	772	46101	188	
	796	41664	907	
	797	38203	799	
	798	29226	314	
	794	22744	965	
L4/C-tail (60-74)				
	801	21034	568	25 (2/8)
	821	63051	651	
	831	118347	17098	
	835	64699	20262	
	806	46992	296	
	809	65804	714	
	820	82307	800	
	839	49039	970	
C-Tail (66-74)				
	859	67246	15345	38 (3/8)
	863	52190	11388	
	875	63829	10331	
	850	34254	821	
	856	54768	541	
	858	23975	468	
	857	36105	1330	
	874	14079	965	

Table 4. Binding of Moā αBgTX peptide antibodies to αBgTX and peptides, SJL, 85-day bleed, 1:500 diluton

Antigen	Mouse No.	Antibody Binding (net cpm) Peptide	αBgTX	% (and No./total) antisera that bind to BgTX
L1(3-16)				
	228	91555	0	25 (2/8)
	212	52940	10572	
	217	49814	4238	
	201	89049	698	
	214	118961	829	
	486	80581	474	
	221	76059	220	
	226	62156	0	
L1/N-tail (1-16)				
	241	41976	5407	33 (3/9)
	247	98706	2978	
	249	58672	8001	
	253	48260	110	
	237	56448	489	
	239	81104	0	
	240	66222	82	
	490	62172	11	
	256	46085	598	
L2(26-42)				
	265	36768	16344	44 (4/9)
	267	54674	20049	
	269	62065	39862	
	271	52750	18545	
	285	19260	0	
	280	122070	16	
	284	73177	0	
	281	105835	359	
	270	64481	1435	

Table 4 (continued)

Antigen	Mouse No.	Antibody Binding (net cpm) Peptide	αBgTX	% (and No./total) antisera that bind to BgTX
L3 (48-59)				
	317	41237	2106	20 (2/10)
	318	52254	4686	
	296	26491	376	
	298	61524	1514	
	310	34332	629	
	314	53788	150	
	315	21078	467	
	323	38843	610	
	325	27761	149	
	326	29235	52	
L3/Ext. (45-59)				
	329	21607	729	25 (2/8)
	342	62439	14243	
	331	59191	9278	
	333	23735	848	
	343	35656	288	
	351	49536	617	
	392	41639	888	
	356	55831	0	
L4/C-tail				
	361	109195	15682	20 (2/10)
	363	74045	18891	
	503	154186	1008	
	505	42169	230	
	501	37090	0	
	508	79866	1146	
	509	64417	561	
	529	83179	0	
	526	50852	0	
	527	42951	501	
C-tail (66-74)				
	414	61237	25997	33 (3/9)
	403	48322	12254	
	415	59152	13124	
	398	39051	410	
	399	51995	338	
	400	35866	84	
	404	60377	254	
	405	28593	306	
	393	14945	1793	

Table 5. Proliferative response of peptide-primed Balb/c AnNcr lymph node cells to challenge in vitro with peptide or the intact BgTX

	Priming peptides (CPM)						
Challenge with:	L1	L1/N-tail	L2	L3	L3/Ext	L4/C-tail	C-tail
Peptide	5864	24586	25028	18632	29067	28501	39869
Opt. dose	(6)	(3)	(12)	(12)	(3)	(25)	(25)
BgTX	5633	6895	13250	13987	26949	15231	4684
Opt. dose	(3)	(12)	(6)	(12)	(1.5)	(3)	(6)
Controls							
Con A	96125	78572	138524	49807	110601	133505	78962
Dose	(1)	(1)	(1)	(1)	(1)	(1)	(1)
R.L1/N-tail	1519	2165	2249	3126	1211	2685	730
Opt. dose	(50)	(25)	(3)	(25)	(12)	(50)	(3)
R.L2	1853	1954	982	1965	3184	3648	2247
Opt. dose	(25)	(12)	(100)	(3)	(6)	(12)	(3)
R.L3/Ext	1897	2365	3156	2978	2106	3378	3492
Opt. dose	(12)	(50)	(3)	(1.5)	(25)	(3)	(12)
Mb	1885	2047	495	2739	2505	2796	3364
BSA	2516	1465	659	2157	2675	2644	2445
None(medium)	2815	2145	621	2552	1464	2968	2634

Table 6. Proliferative response of peptide-primed SJL/JCr lymph node cells to challenge in vitro with peptide or intact BgTX

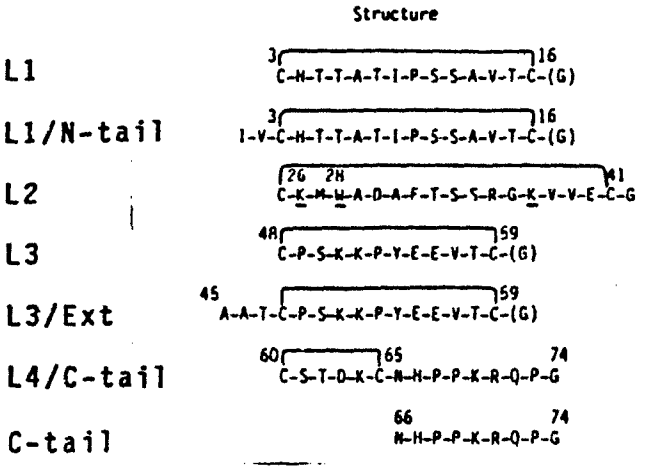
Challenge with:	Priming peptides (CPM)						
	L1	L1/N-tail	L2	L3	L3/Ext	L4/C-tail	C-tail
Peptide	23382	8421	24339	4561	4826	41450	20254
Opt. dose	(1.5)	(25)	(25)	(1.5)	(12)	(50)	(25)
BgTX	18482	3476	9845	3380	2670	30831	12614
Opt. dose	(12)	(25)	(25)	(6)	(3)	(25)	(12)
Cotrols							
Con A	87834	64879	92565	58647	74869	117065	98521
Dose	(1)	(1)	(1)	(1)	(1)	(1)	(1)
R.L1/N-tail	2205	1258	2964	1875	1211	1161	518
Opt. dose	(25)	(1.5)	(6)	(12)	(50)	(3)	(100)
R.L2	3456	3101	1490	2864	1865	3658	3865
Opt. dose	(6)	(12)	(25)	(6)	(3)	(12)	(6)
R.L3/Ext	2871	1902	3346	1642	2402	3351	2269
Opt. dose	(3)	(12)	(1.5)	(50)	(12)	(3)	(25)
Mb	1990	1984	1855	3147	2645	2329	2041
BSA	2218	1216	1529	2589	2987	3257	1415
None(medium)	2630	1461	1039	2163	2891	2096	1523

Table 7. Protection of mice against BgTX by immunization with BgTX or with synthetic BgTX peptides.

	Protection parameters for Balb/c and SJL mice			
	Balb/c		SJL	
Immunizing Antigen	LD_{50} (μg BgTX/mouse)	Protection Index	LD_{50} (μg BgTX/mouse)	Protetion Index
None or random peptides[+]	3.20	1.00	3.60	1.00
L1	10.27	3.21	8.86	2.46
L1/N-tail	8.36	2.61	7.86	2.18
L2	10.27	3.21	9.76	2.71
L3	7.86	2.46	7.94	2.21
L3/Ext	8.36	2.61	8.57	2.38
L4/C-tail	8.36	2.61	8.64	2.40
C-tail	10.27	3.21	8.86	2.46
BgTX	31.0	9.69	26.50	7.36
Mixture L1, L2, C-tail	14.63	4.57	nd	nd
Multi-Peptide Conjugate of L1, L2, C-tail	>57.8	>18.1	nd	nd

[+]This group includes 45 unimmunized mice and mice that were immunized with randomized sequence peptides R1-16 (45 mice), R26-41 (45 mice), and R45-59 (45 mice).

Covalent Structure of the Synthetic BgTX Peptides



Covalent structure of the Randomized Sequence Analogs of the BgTX Peptides

R.L1/N-tail	T.H.C.I.T.V.A.S.T.P.I.T.S.V.A.C.G
R.L2	C.W.V.R.D.T.A.M.F.K.G.A.K.S.E.V.S.C.G
R.L3/Ext	K.S.P.C.A.Y.K.E.P.E.T.T.V.A.C.G

Fig.1. Structures of the synthetic peptides representing the BgTX loops and exposed regions and three peptide analogs which had the same amino acid composition as the respective peptides L1/N-tail, L2 and L3/Ext but whose sequences were randomized.

Fig.2. Binding of Rabbit anti-BgTX antibody to BgTX peptides

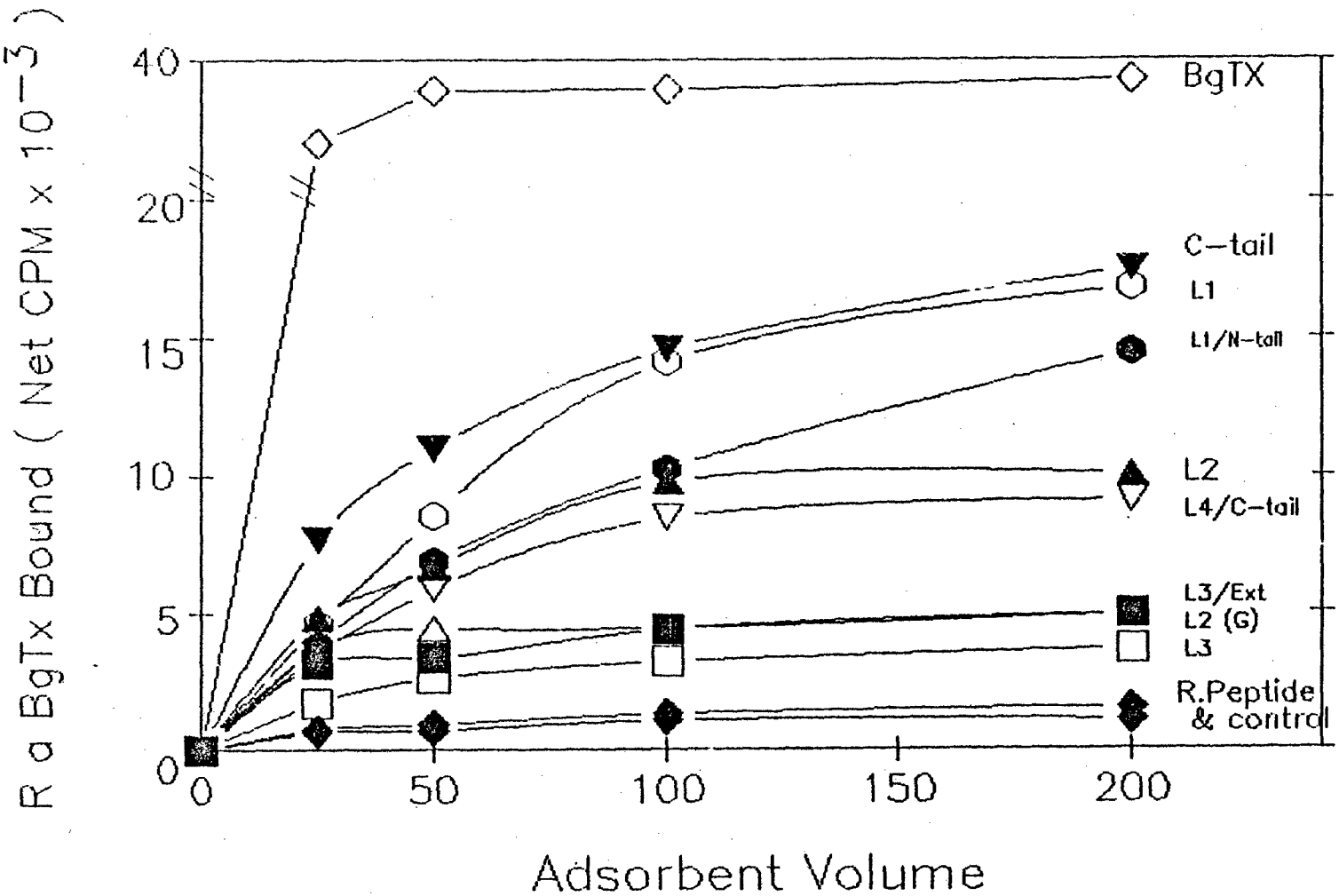


Fig.3. Rabbit anti-BgTX antibidy binding to BgTX peptides

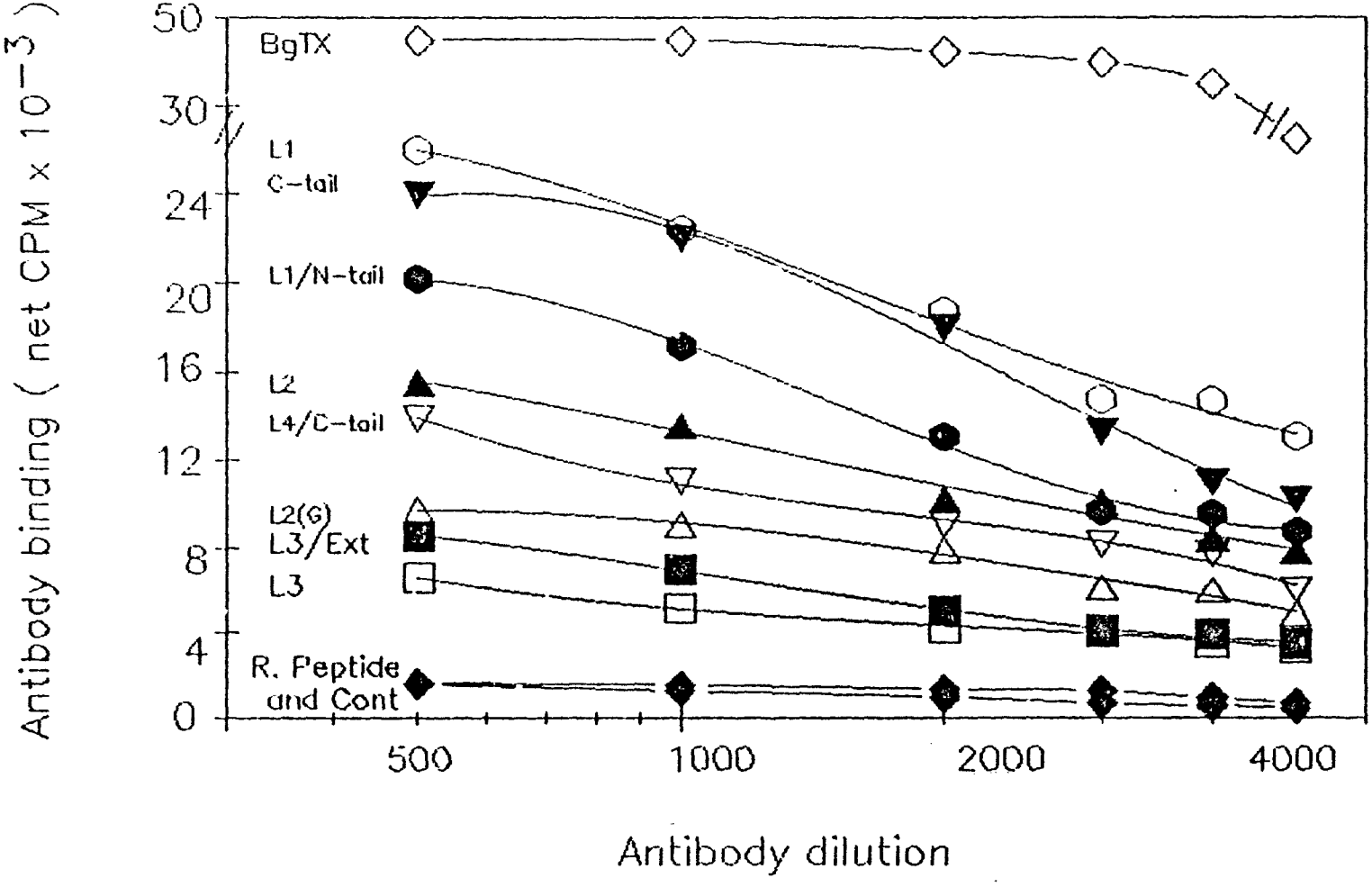


Fig.4. Binding of Mouse anti-BgTX antibodies to BgTX peptides

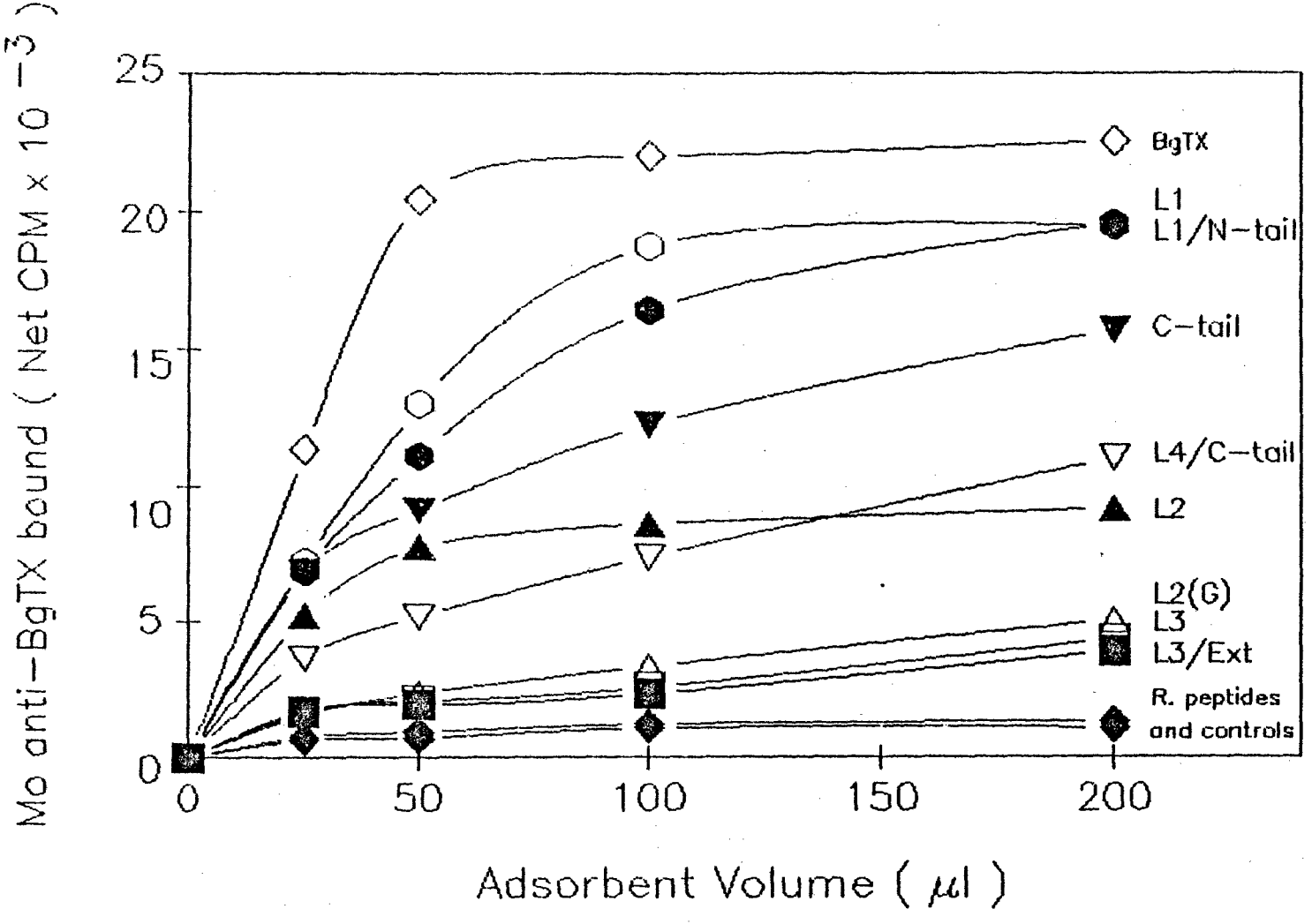


Fig.5. Proliferative response of lymph node cells from Balb/C AnNC to opt. dose of BgTX or peptides

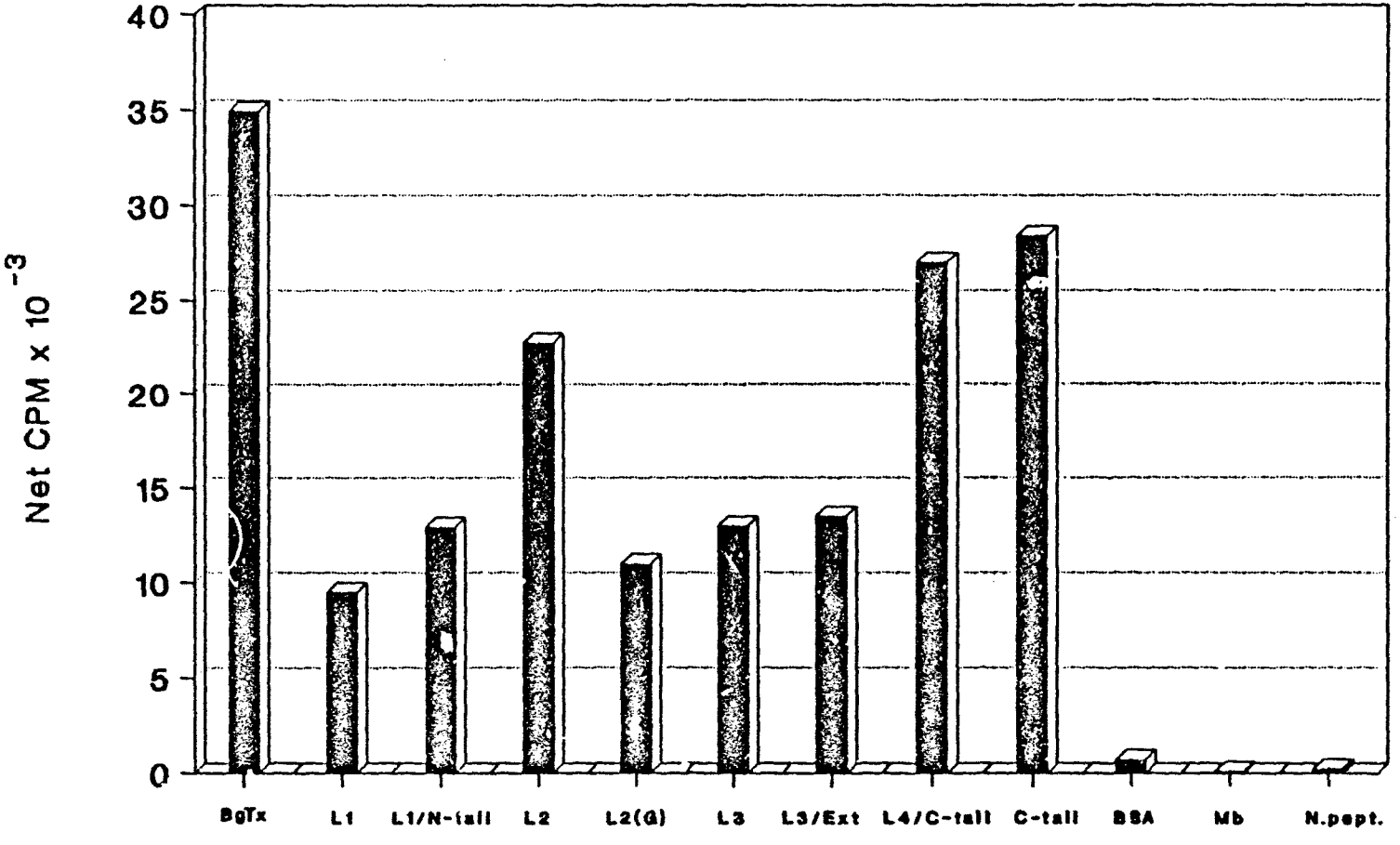


Fig.6. Proliferative response of lymph node cells from CBA/JCr to opt. dose of BgTX or peptides

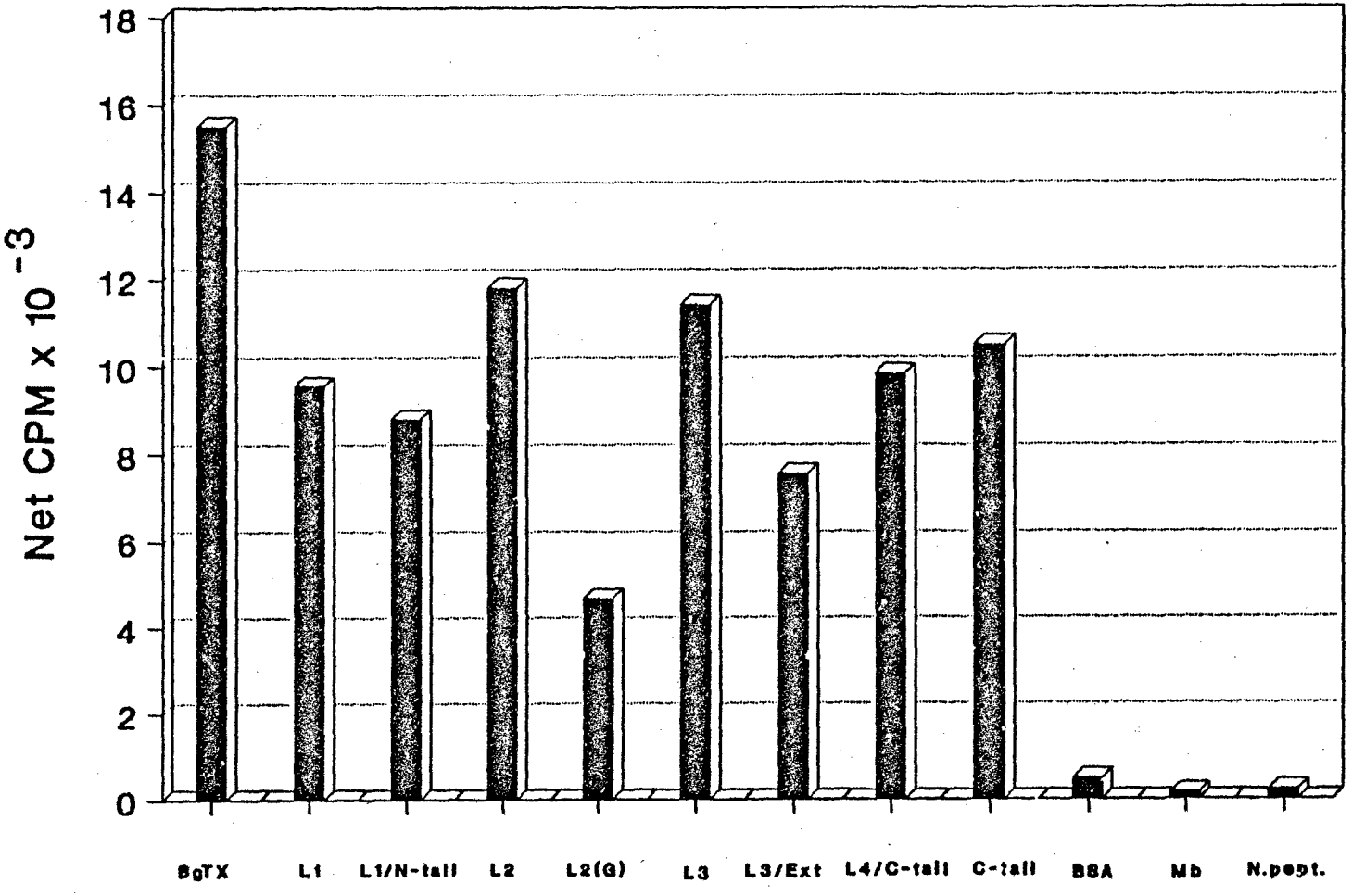


Fig.7. Proliferative response of lymph node cells from C3H/HeNCr to opt. dose of BgTx or peptides

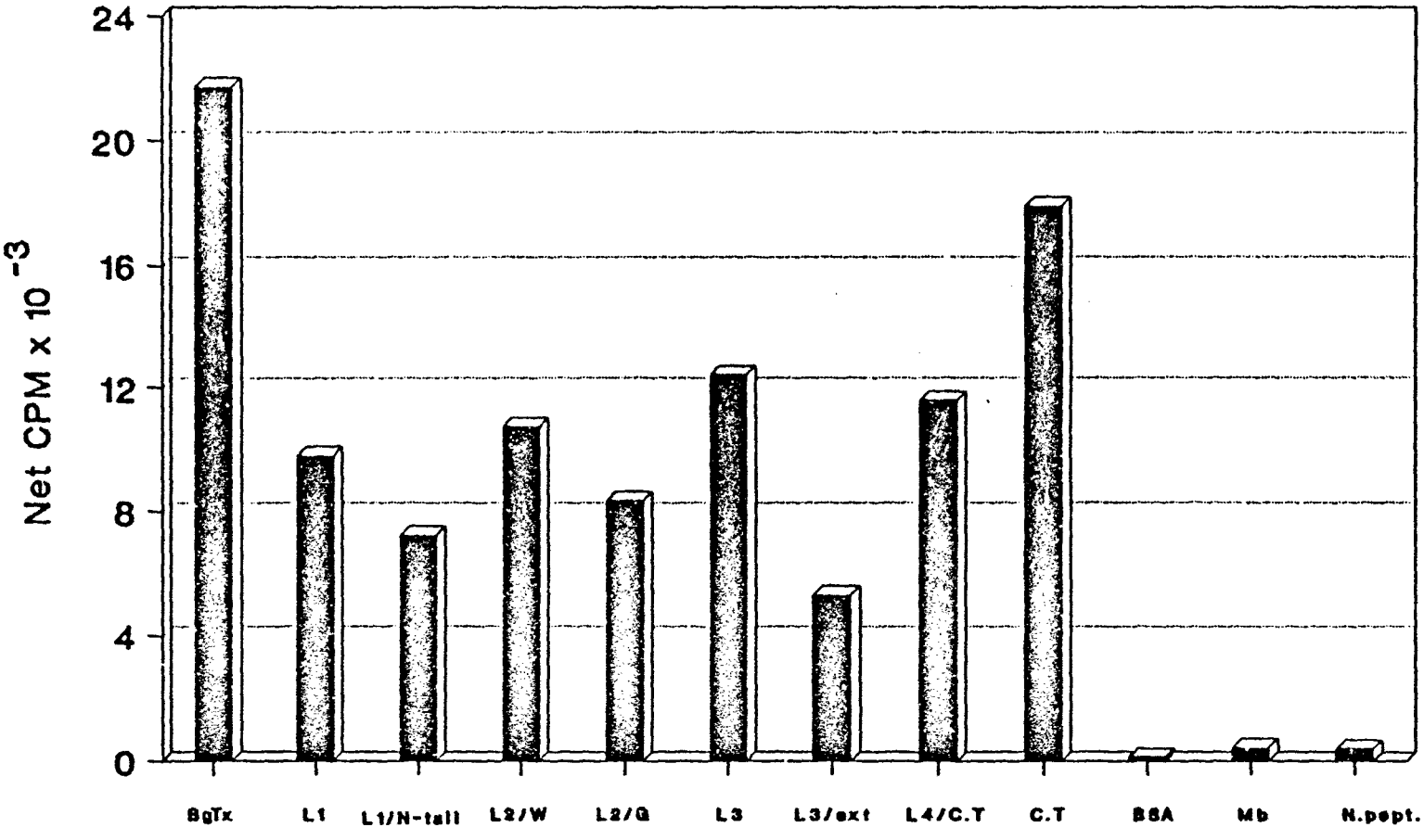
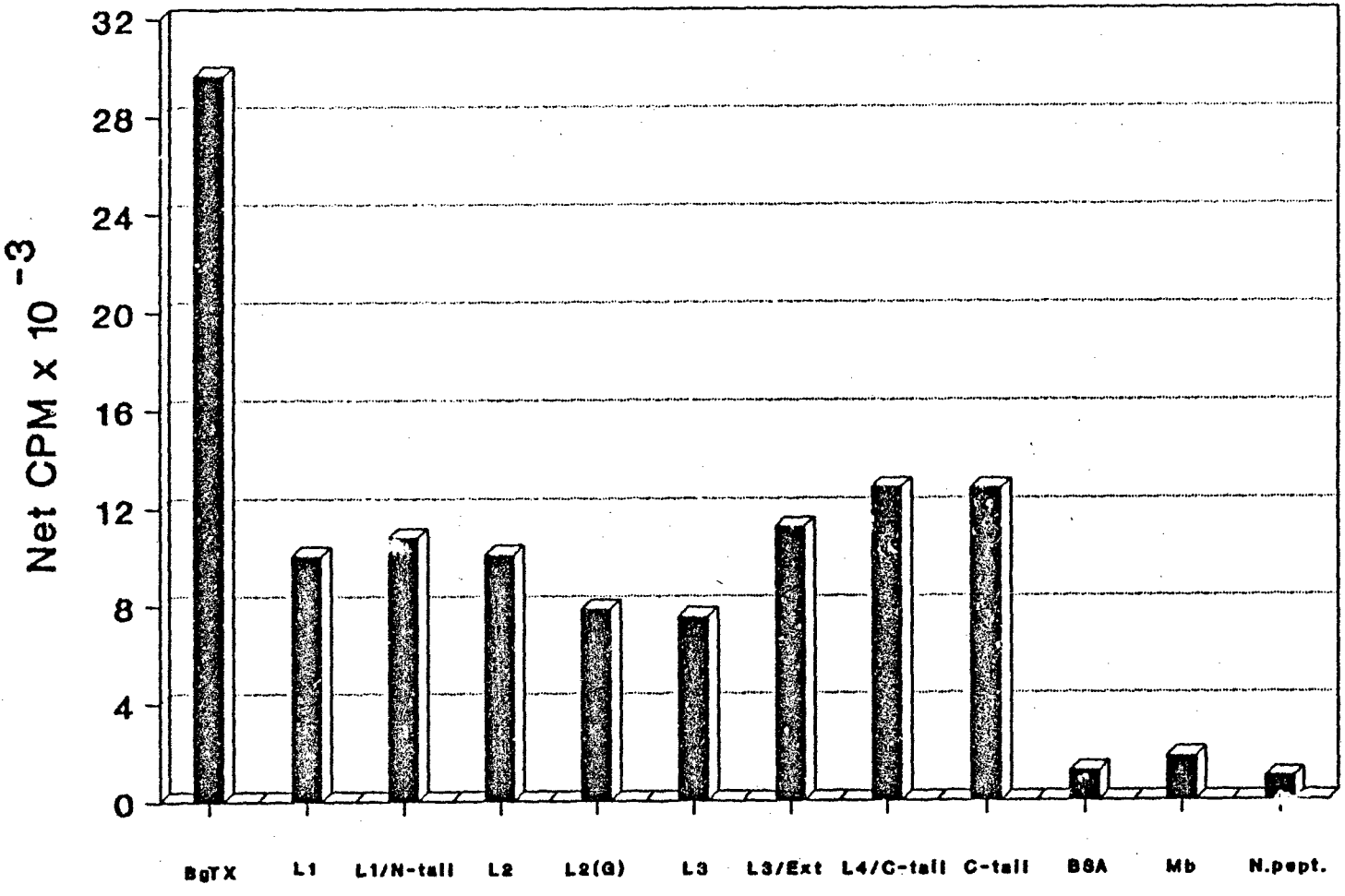


Fig.8. Proliferative response of lymph node cells from C57/BL(H-2^b) to opt. dose of BgTX or peptides



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July 16, 1992

FILE: BAYM:004

VIA FACSIMILE / CONFIRMATION BY MAIL

Dr. M. Zouhair Atassi
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030

Re: Synthetic Toxins and Methods of Use
Drs. Atassi, Manshouri, and McDaniel

Dear Dr. Atassi:

We have received authorization from Mr. Crocker of Baylor College of Medicine to proceed with filing of the above-referenced patent disclosure. I will incorporate the materials newly disclosed to us into your previously disclosed data. You may expect a draft of the application soon.

By way of reminder, I would like, once again, to caution you concerning the publication of any of these data prior to the filing of a patent application with the U.S. Patent and Trademark Office. While it is possible in the U.S. to get patent protection on work published less than a year in advance of the filing date of such a patent application, most foreign jurisdictions require absolute novelty and would not allow any claims to published materials.

Please keep me advised of any publications you are considering.

Very truly yours,

Steven McDaniel by dac

C. Steven McDaniel

I:\baym\004\ltr\01.mcd

cc: Sam Crocker, Esq.
Lynne Downs
Charles DeLaGarza, Esq.
Melinda Patterson, Esq.

Fig.9. Proliferative response of lymph node cells from SJL(H-2^{b}) to opt. dose of BgTX or peptides

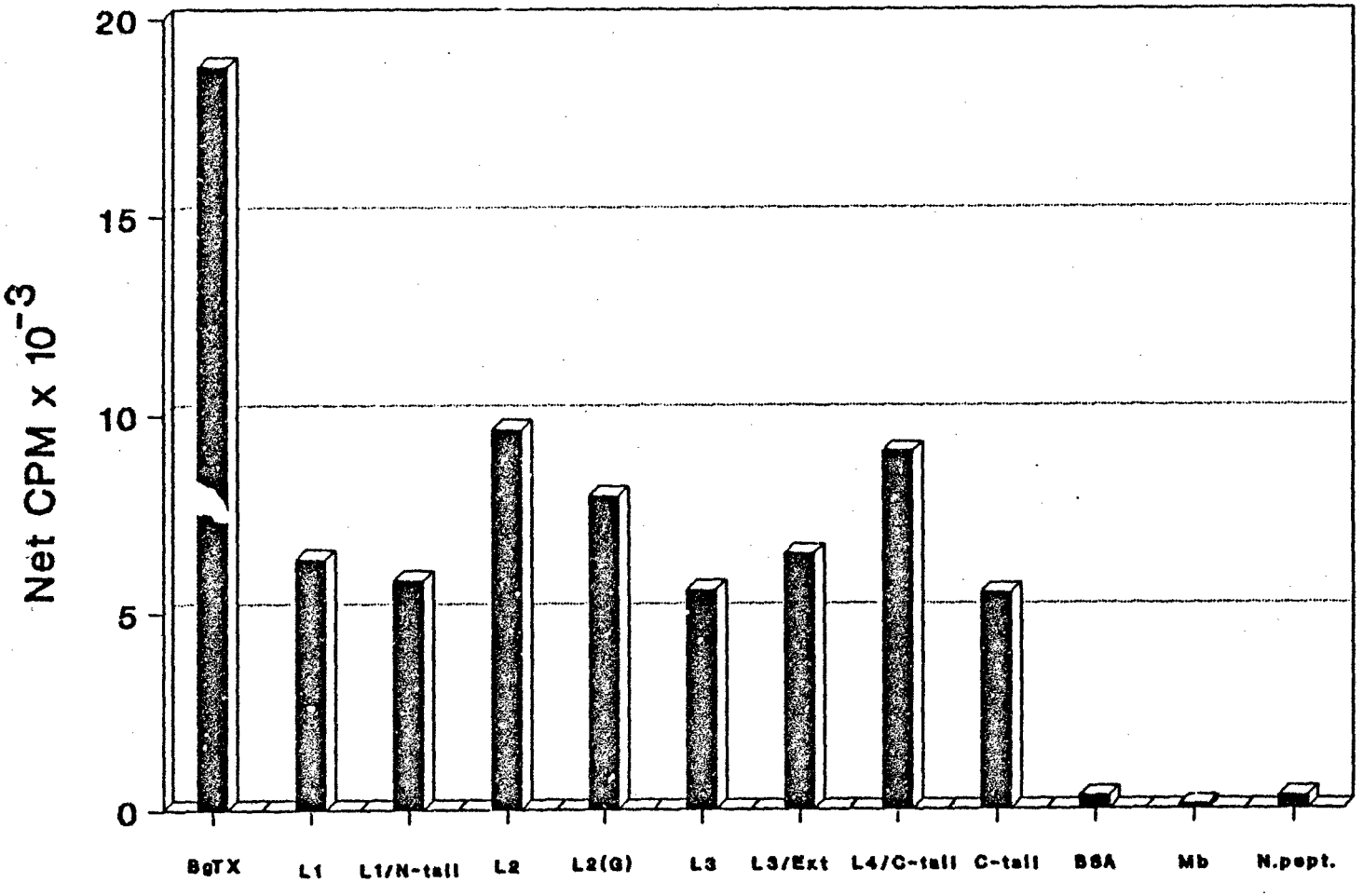

Fig. 10. Binding of Mouse (Balb/C) anti-BgTX antibodies to BgTX peptides

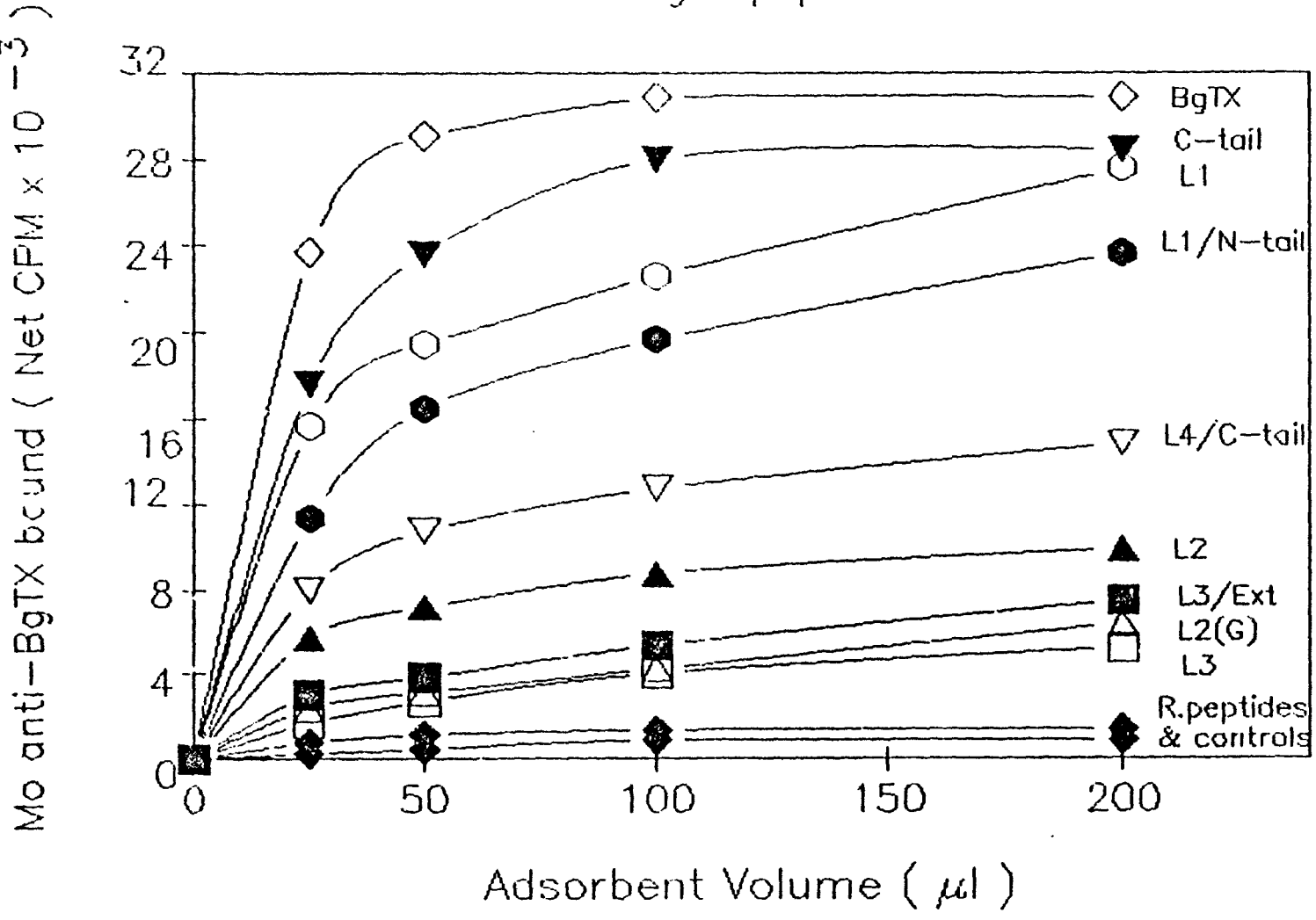

Fig.11. Binding of Mouse (C57/BL6) anti-BgTX antibodies to BgTX peptides

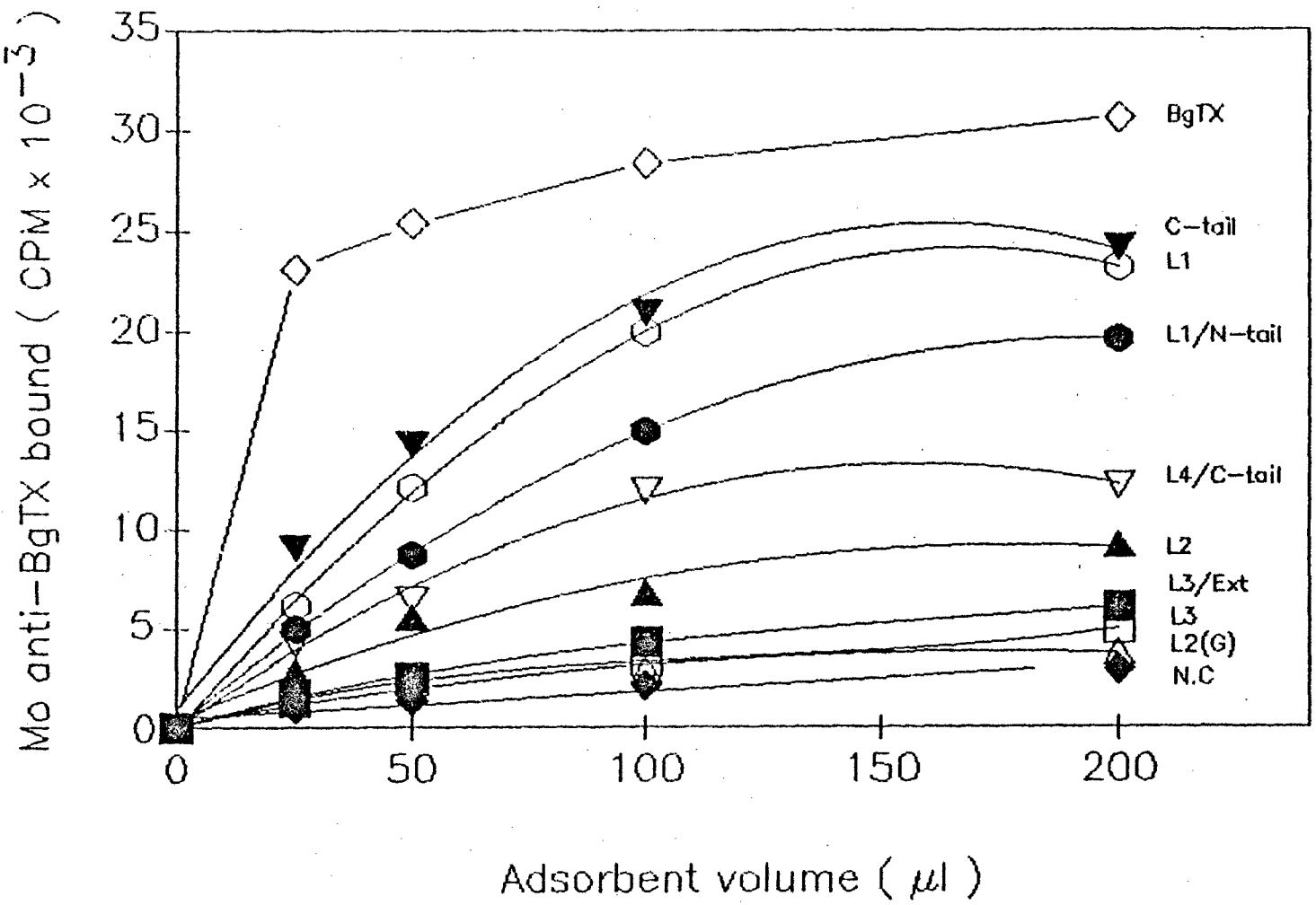

Fig. 12. Binding of Mouse (3oL) anti-BgTX antibodies to BgTX peptides

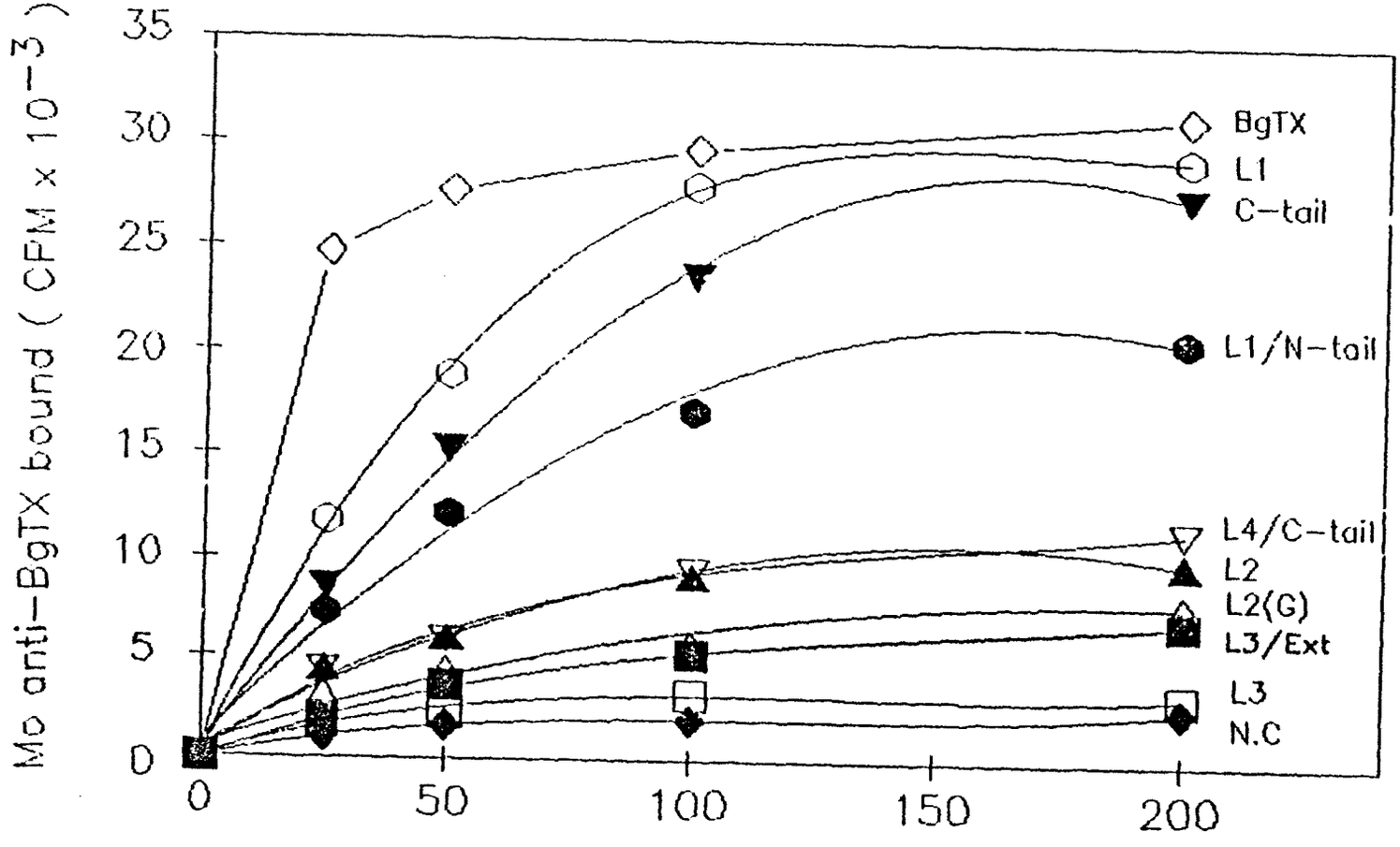

Fig.13. Proliferative response of lymph node cells from Balb/C AnNCr(H-2^d) mice that were primed with L3/Ext to challenge invitro with L3/Ext, intact BgTX and R.L3/Ext

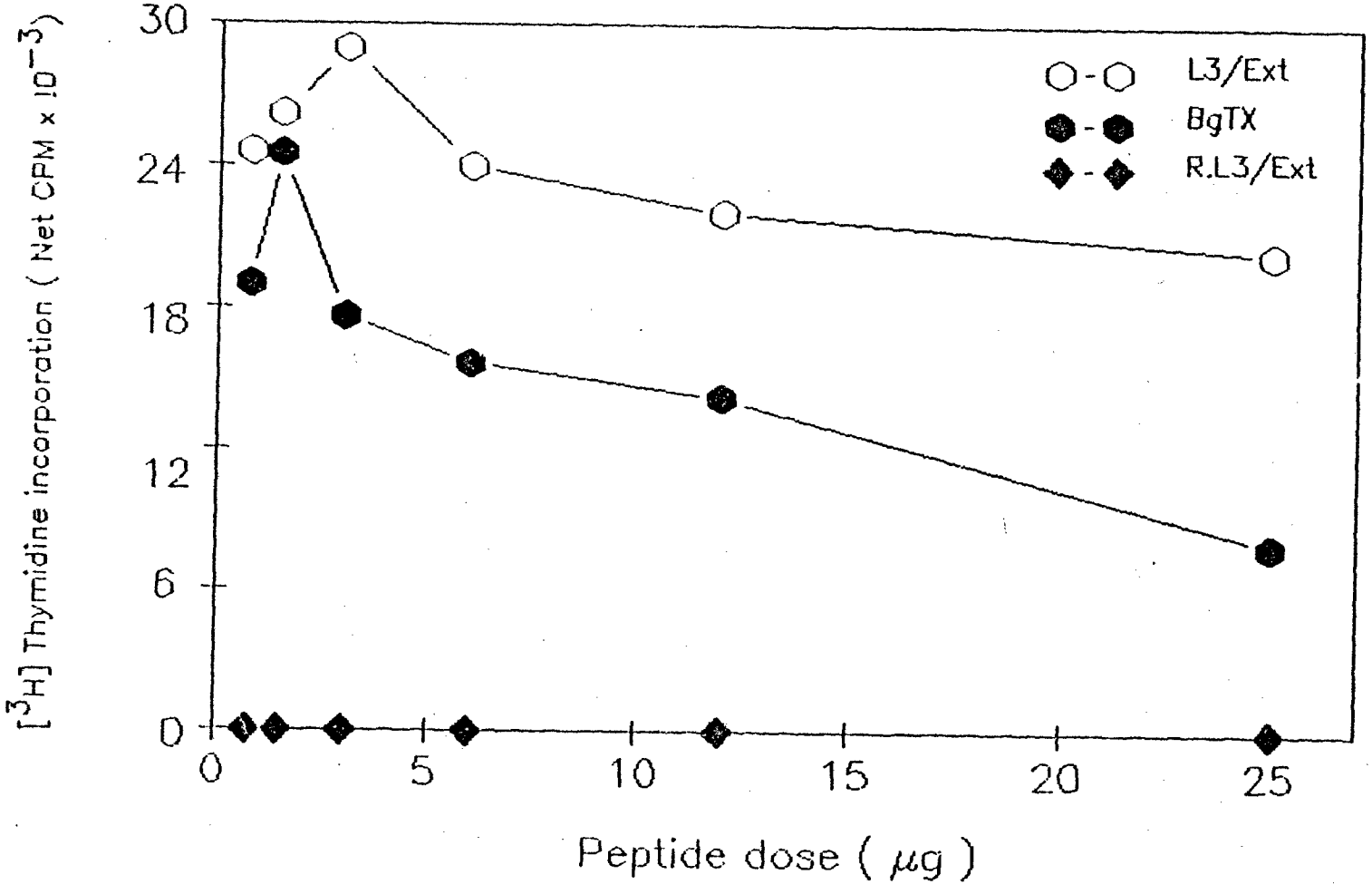

Fig.14. Proliferative response of lymph node cells from SJL/JCr(H-2^5) mice that were primed with L4/C-tail to challenge in vitro with L4/C-tail, intact BgTX and R.L2

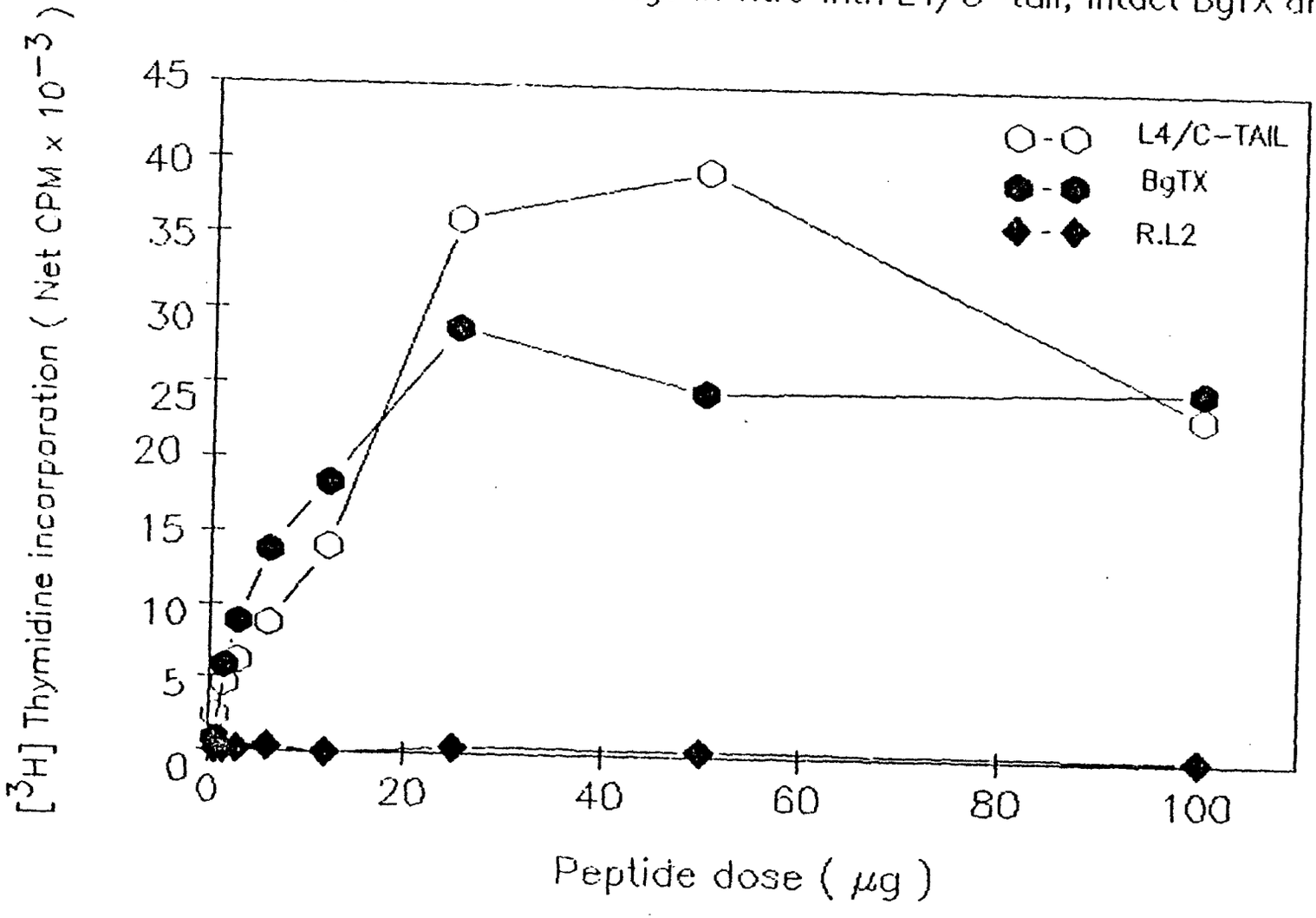

Fig.15. Proliferative response of lymph node cells from peptide - primed Balb/c mice to challenge with peptide or intact aBgTx

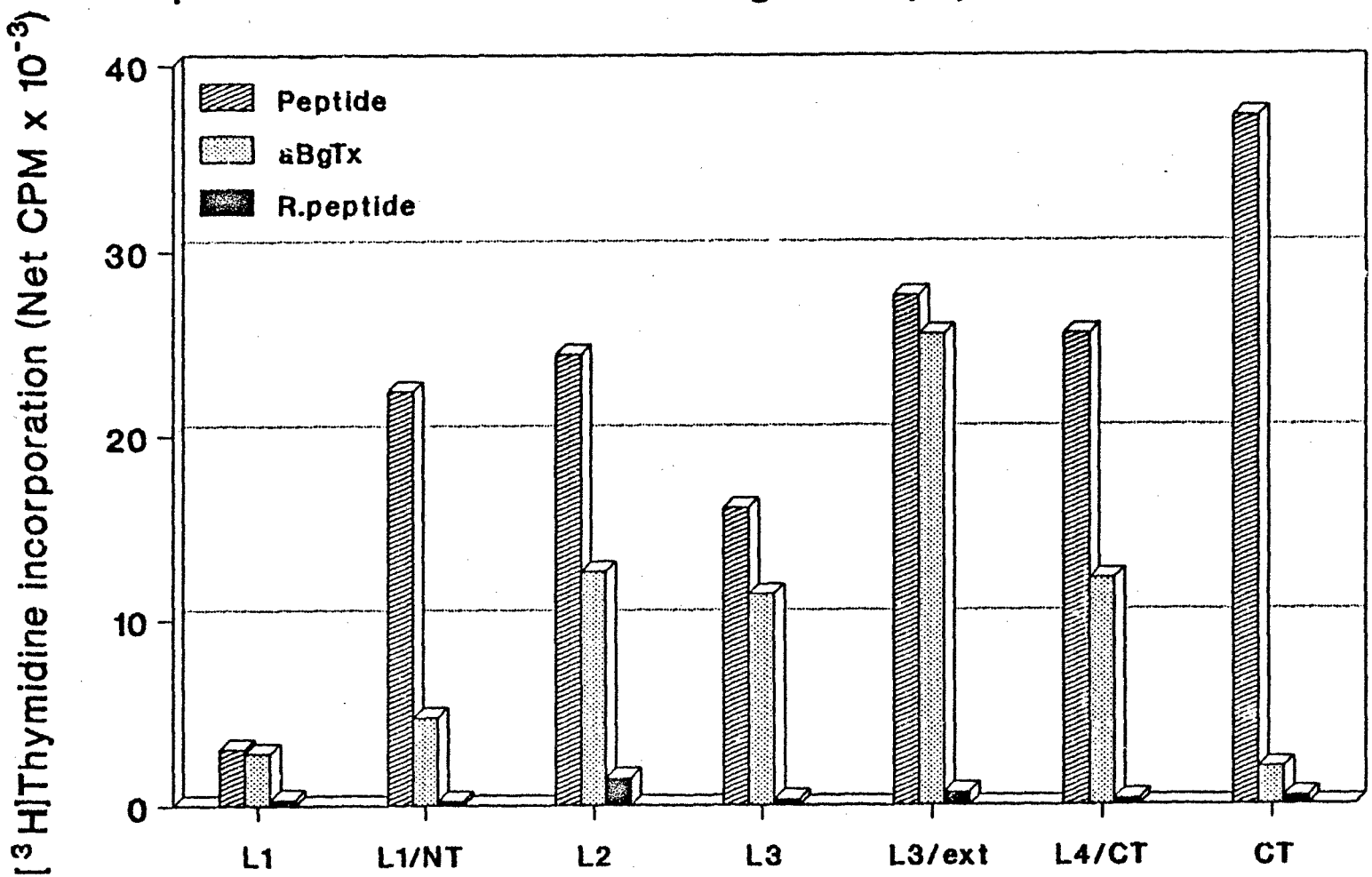

Fig.16. Proliferative response of lymph node cells from peptide -primed SJL mice to challenge with peptide or intact aBgTx

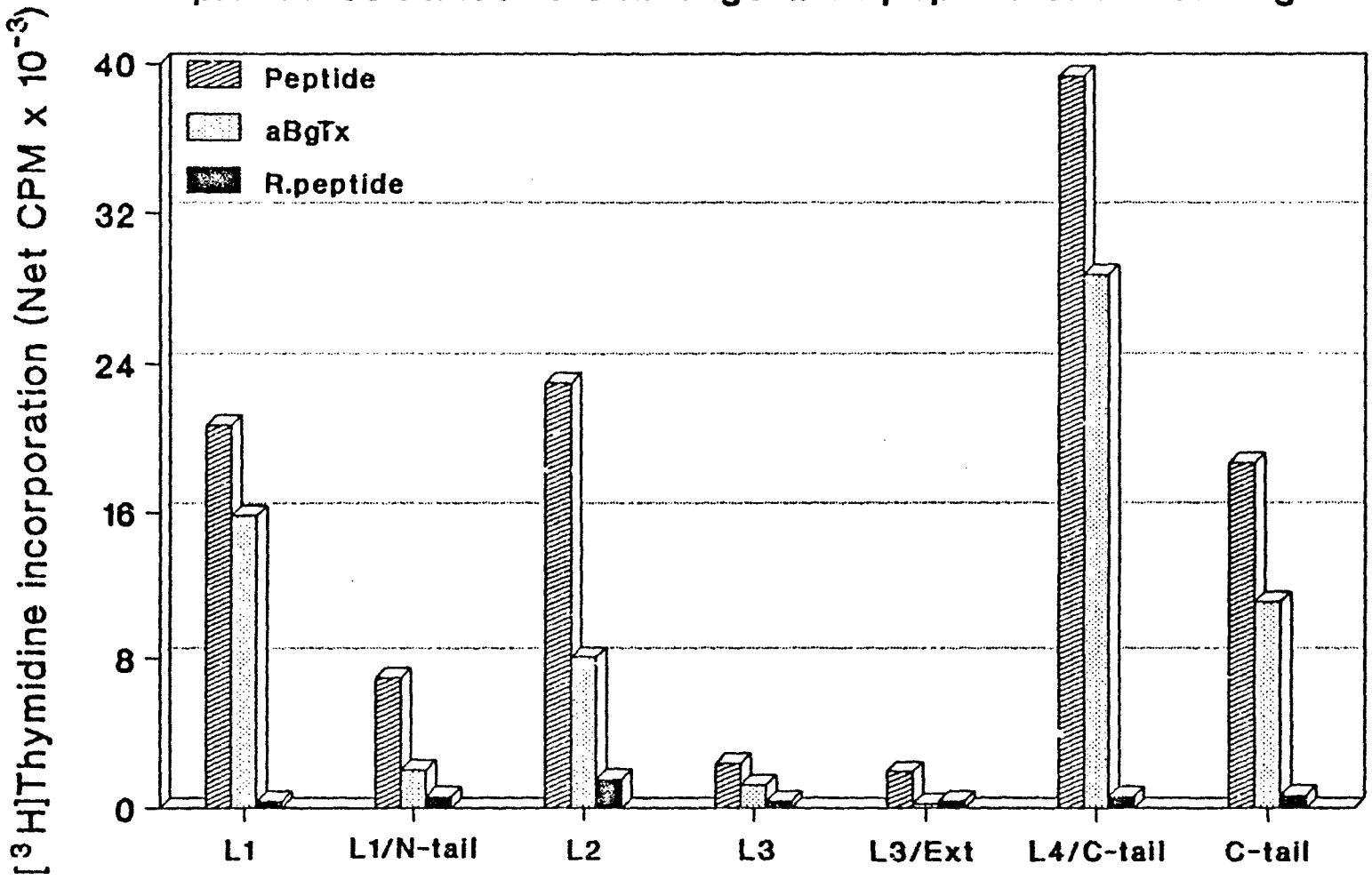

Fig.17. Survival to BgTX challenge of Balb/C mice which were immunized with synthetic BgTX peptides

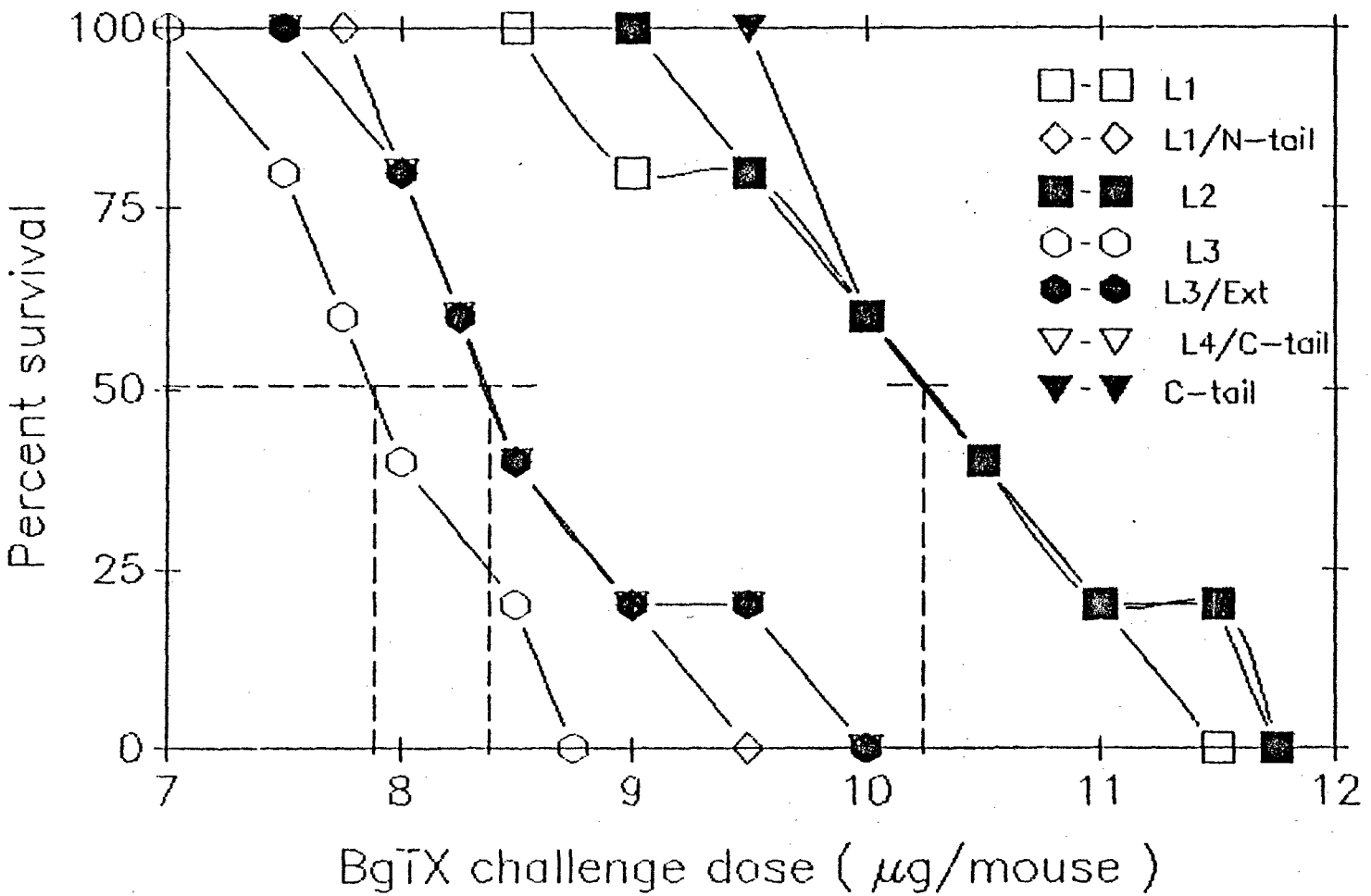

Fig. 16. Survival to BgTX challenge of Balb/C mice which were unimmunized or immunized with BgTX or synthetic peptide L2

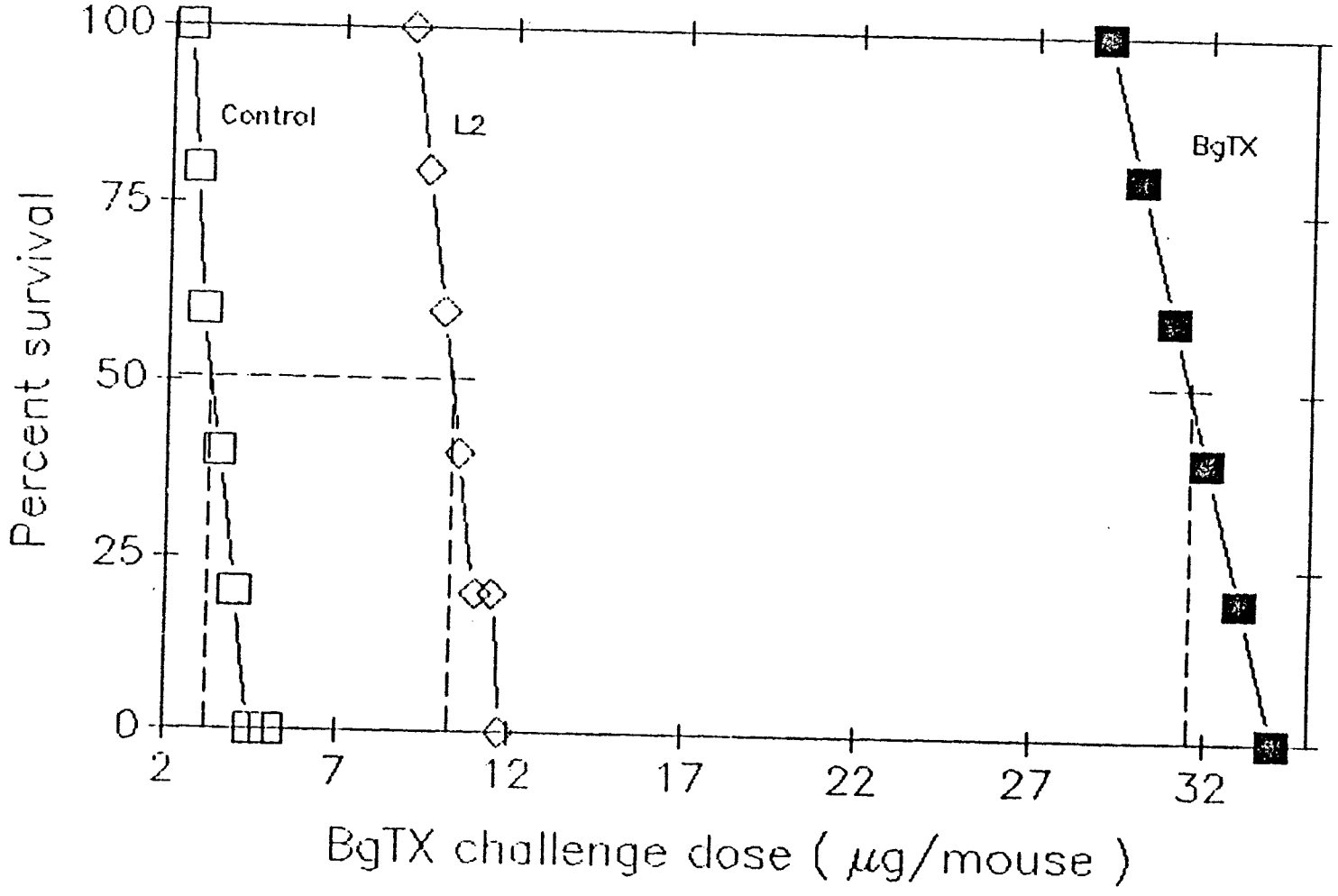

Fig. 19. Survival to BgTX challenge of SJL mice which were immunized with synthetic BgTX peptides

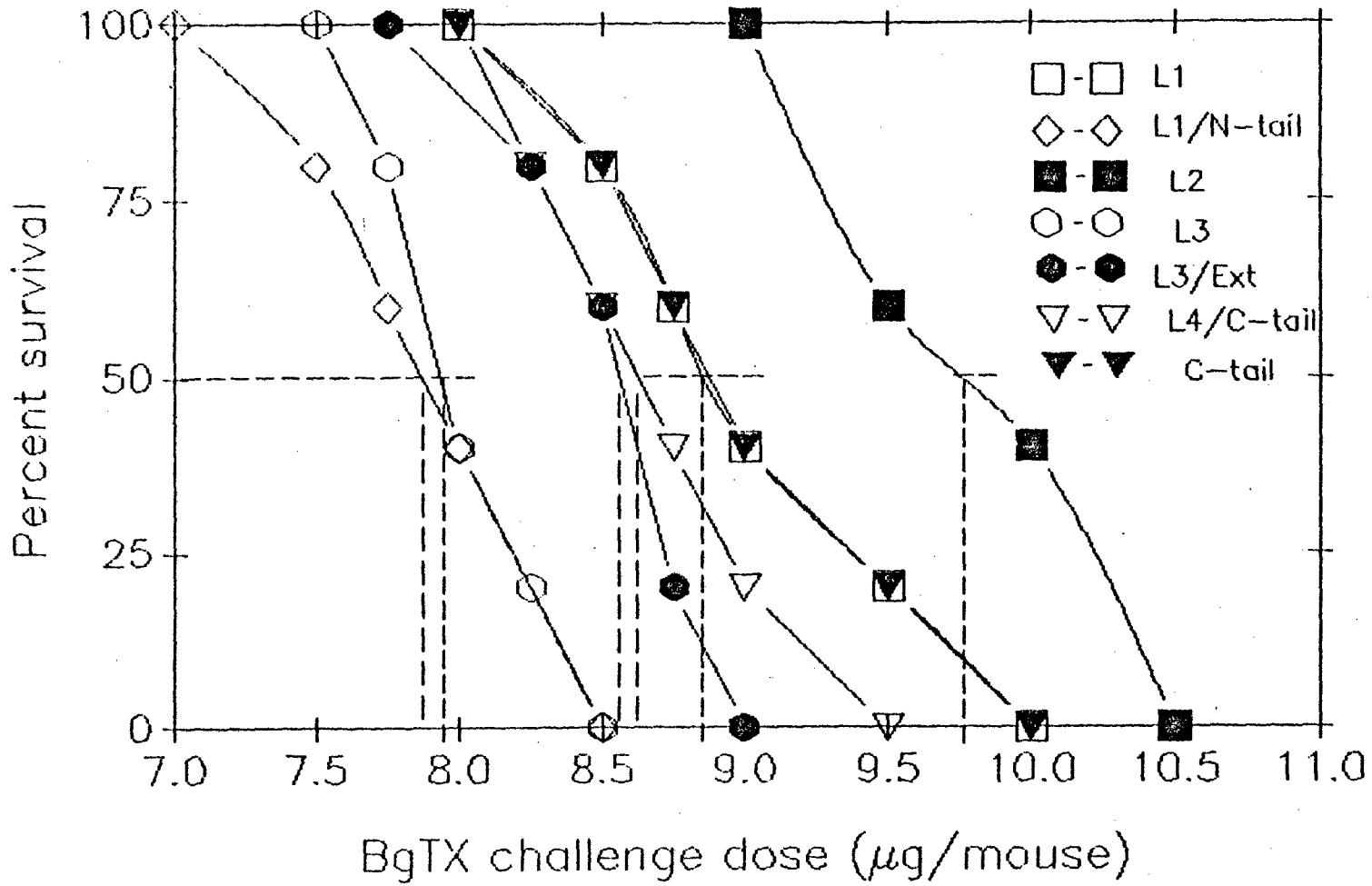

Fig.20. Survival to BgTX challenge of SJL mice which were unimmunized or immunized with BgTX or synnthetic peptide L2

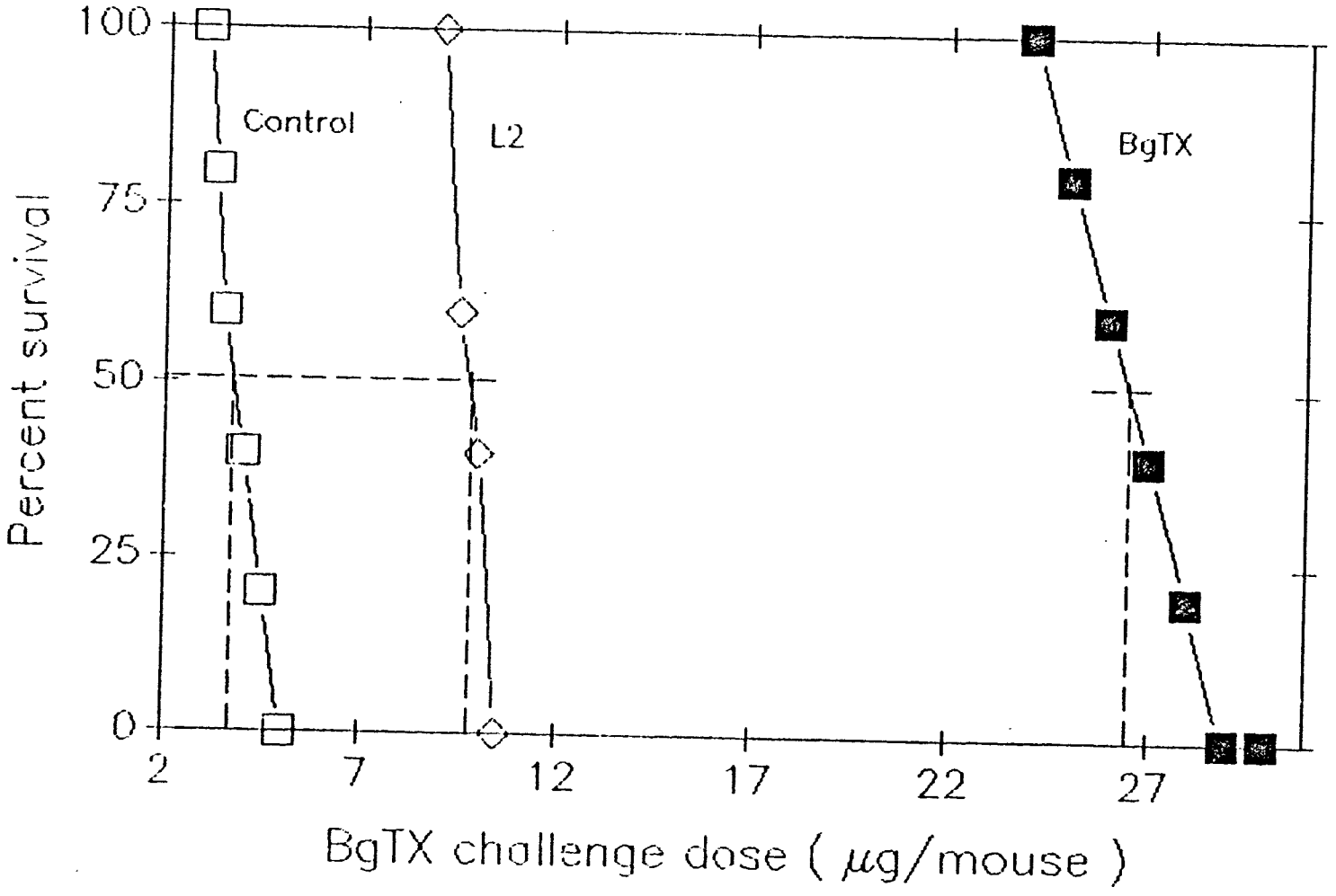

Fig.21. Relationship between outcome of challenge with BgTX and levels of antibodies that bind to BgTX in mice that had been immunized with an equimolar mixture of peptides L1, L2 and C-tail or with a conjugate of the three peptides to ovalbumin.