are the absence of the first 12 residues at the $NH_2$-terminal end in calmodulin and a D-E linker shortened by three residues. The four $Ca^{2+}$ sites in calmodulin have binding constants ($K_a \simeq 10^5 M^{-1}$) similar to the lower affinity, $Ca^{2+}$ specific sites in the $NH_2$ domain of TnC (*29*). Whether calmodulin assumes a structure similar to that of TnC and undergoes similar structural perturbations upon metal binding remains to be determined.

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### References

1. P. C. Leavis and J. Gergely, *CRC Crit. Rev. Biochem.* **16**, 235 (1984).
2. M. L. Greaser and J. Gergely, *J. Biol. Chem.* **246**, 4226 (1971).
3. ———, *ibid.* **248**, 2125 (1973).
4. J. D. Potter and J. Gergely, *ibid.* **250**, 4628 (1975).
5. J. H. Collins, J. D. Potter, M. J. Horn, G. Wilshire, N. Jackman, *FEBS Lett.* **36**, 268 (1973).
6. G. M. Strasburg, M. L. Greaser, M. Sundaralingam, *J. Biol. Chem.* **255**, 3806 (1980).
7. G. M. Strasburg, M. L. Greaser, S. T. Rao, M. Sundaralingam, in *Calcium-Binding Proteins and Calcium Function*, F. L. Siegel *et al.*, Eds. (Elsevier North-Holland, New York, 1980), p. 289.
8. The method has been implemented into an integrated program package called ISIR-ISAS process, was reported at the 13th International Union of Crystallography Congress held at Hamburg, Germany, 9 to 18 August 1984. [See B. C. Wang *et al.*, *Acta Cryst.* **40A**, C12 (1984)]. An early objective of the method was to resolve the phase ambiguity problem in a single isomorphous derivative without anomalous scattering information or in a single wavelength anomalous scattering data set. A brief description of this application is also presented in *Diffraction Methods in Biological Macromolecules* (a volume of *Methods of Enzymology*), H. Wyckoff, Ed. (Academic Press, New York, in press.)
9. O. Herzberg, K. Hayakawa, M. N. G. James, *J. Mol. Biol.* **172**, 345 (1984).
10. O. Herzberg and M. N. G. James, *Acta Cryst.* **40A**, C37 (1984). Abstract 02.1-40 of paper presented at the XIII International Union of Crystallography Congress, Hamburg, Federal Republic of Germany, 9 to 18 August (1984).
11. For example, see J. Hogle *et al.*, *Acta Cryst.* **B37**, 591 (1981).
12. The difference Patterson maps were calculated with coefficients $(|F_D| - |F_N|)^2$ where $D$ and $N$ refer to the derivative and native structure amplitudes, respectively.
13. The data set on Nd extended to 3 Å resolution and on Au to 4-Å resolution. However, useful phasing power extended only to 3.8 Å and 5 Å, respectively.
14. D. M. Blow and F. H. C. Crick, *Acta Cryst.* **12**, 794 (1959).
15. B. C. Wang, *ibid.*, in press. The method first transforms the original Fourier map into a new map based on the density values contained within a sphere of a certain radius around each point, which depends on the resolution of the map; in the case of TnC at 3.8-Å resolution, it was 10 Å. This new map is related to the probability of finding molecules instead of atoms. The protein solvent boundary is then located from this "probability" map and with the known solvent content of the crystal. In the case of TnC, a value of 40 percent was used. The transformation process, which involves a summation of density from the original Fourier map, invokes the concept that a molecule is an assemblage of atoms; thus for the purpose of locating molecules, the new map is more suitable than the conventional map, which is used for describing individual atoms rather than molecules. The summation process will transform the true signal but not the noise in the map because the noise generally occurs as a higher frequency component in the density. Thus, the noise can be filtered out by the summation process. Therefore, such a transformation can produce a map with an error-free signal of the molecule and can allow an accurate determination of the protein-solvent boundary even when a high level of noise is present in the original map.
16. The starting phase angles for the 1407 reflections having no previous phase information were arbitrarily set to 0°.
17. The molecules are packed with the long helix lying over the internal $3_2$-axis of the unit cell. The $NH_2$- and COOH-domains of the $3_2$-axis related molecules are tightly packed and are in close contact with each other and with other symmetry-related molecules. This tight packing made it difficult to carve out the molecular boundary in our earlier work at 3.8-Å resolution which resulted in tracing the molecular boundary incorporating the $NH_2$- and COOH-domains of two different molecules. After the phase improvement and extension, not only was the backbone trace clear, but also the intermolecular boundary.
18. R. H. Kretsinger and C. D. Barry, *Biochim. Biophys. Acta* **405**, 40 (1975).
19. R. H. Kretsinger and C. E. Nockolds, *J. Biol. Chem.* **248**, 3313 (1973).
20. The EF-hand describes the nearly perpendicular arrangement of the E and F helices flanking the 12-residue $Ca^{2+}$-binding loop, in analogy to the stretched out right hand with the forefinger (E helix) and thumb (F helix) and the remaining fingers folded to form the $Ca^{2+}$-binding loop. The terminology was first used to describe the $Ca^{2+}$-binding helix-loop-helix configuration in parvalbumin (*19*).
21. D. M. E. Szebeny, S. K. Obendorf, K. Moffat, *Nature (London)* **294**, 327 (1981).
22. It is conceivable that the $NH_2$-domain assumes a different helix-loop-helix configuration on $Ca^{2+}$ binding. Circular dichroism data (*2, 3*) indicate that, while $Ca^{2+}$ binding to the COOH-domain results in a large increase in helix content, the same is not true for the $NH_2$-domain. This may simply mean that the expected helices of the $NH_2$-domain are already preformed in the metal-free state and perhaps undergo a tertiary structural rearrangement. In contrast, the increased helix content on $Ca^{2+}$ binding to the COOH-domain has been attributed to the formation of the E and G helices (*2, 3*). It is also possible that the D-E linker helix found in the x-ray structure is formed on $Ca^{2+}$ binding to the COOH-domain, and contributes to the exhanced helix content of the molecule.
23. In parvalbumin and intestinal $Ca^{2+}$-binding protein, the $Ca^{2+}$-binding loops differ in glycine content. The lower $K_a$ values ($10^5 M^{-1}$) are probably associated with the presence of more than one glycine in the $Ca^{2+}$-binding loops, whereas the loops with higher $K_a$ values ($10^7 M^{-1}$) are associated with the presence of only one glycine. In calmodulin, all four loops contain two glycines and show lower $K_a$ values for the four sites.
24. For example, see J. Gergely and P. C. Leavis, in *Muscle Contractions: Its Regulatory Mechanisms*, S. Ebashi *et al.* Eds. (Springer-Verlag, Berlin, 1980), p. 191.
25. P. C. Leavis, J. Gergely, Z. Grabarek, W. Drabikowski, B. A. Levine, in *Calcium-Binding Proteins and Calcium Function*, F. L. Siegel *et al.*, Eds. (Elsevier North-Holland, New York, 1980), p. 271.
26. Many of the biophysical observations on TnC and its fragments in solution are consistent with the x-ray structure. See also B. A. Levine, D. Mercola, D. Coffman, J. M. Thornton *J. Mol. Biol.* **115**, 743 (1977); K. B. Seamon, A. J. Hartshorne, A. A. Bothner-By, *Biochemistry* **16**, 4039 (1977).
27. The combined hydrophobic and ionic stabilization energies of the $Ca^{2+}$-bound COOH-domain may be expected to be higher than the essentially hydrophobic interaction energy of the metal-free state. In contrast, in the $NH_2$-domain, the hydrophobic interaction energy may be higher in the metal-free state than the combined hydrophobic and ionic interaction energies on $Ca^{2+}$ binding. Better insights on the molecular properties of TnC should be available when the higher resolution structure of TnC, the apo-protein and the four-$Ca^{2+}$ protein become available.
28. W. Y. Cheung, *Science* **207**, 19 (1980).
29. J. D. Potter *et al.*, in *Calcium-Binding Proteins and Calcium Function*, R. H. Wasserman *et al.*, Eds. (Elsevier, Amsterdam, 1977), p. 239.
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## Effects on *Trichinella spiralis* of Host Responses to Purified Antigens

Abstract. *Purification of two antigens (48-kilodalton polypeptide and a group with major subunits of 50 and 55 kilodaltons) from the infective larvae of the parasitic nematode* Trichinella spiralis *was recently reported. Immunization of mice with either of these antigens induces strong resistance to a subsequent challenge infection. In the study reported here the mechanism of this resistance was investigated by monitoring the parasite's life cycle in mice immunized with the antigens. Immunized mice were able to expel intestinal adult worms and to inhibit the fecundity of adult female worms at an accelerated rate compared to control mice. Accelerated expulsion and inhibition of fecundity may account entirely for the level of resistance induced by immunization. Although the effects of the immune response apparently are exerted on adult worms, the target antigens are expressed only by developing larvae. This suggests that immune effector mechanisms act on intestinal larvae in such a way that they develop into defective adults.*

Infection with the parasitic nematode *Trichinella spiralis* stimulates complex, stage-specific immunologic responses in the mammalian host. These responses inhibit development of the parasite at several points in its life cycle. Ultimately, they can limit the production and perhaps the migration of the newborn

larvae (*1*), the stage responsible for the most serious aspects of pathology in the host (*2*). Immunization of the host with certain crude antigen preparations stimulates a protective immune response equally well (*3, 4*). However, little is known of the molecular targets, among the many antigen species presented to the host, of the protective response or of the relation of the target antigens to effects on the parasite.

Recently we described the isolation of three distinct antigens from the infective stage 1 larva of *T. spiralis* by monoclonal antibody affinity chromatography (*5*). Two of these antigens, a 48-kD polypeptide secreted by the beta stichocytes and a group of cross-reacting polypeptides with major subunits of 50 and 55 kD secreted by the alpha stichocytes, induced resistance to subsequent infection when injected into mice (stichocytes are secretory cells that discharge into the worm esophagus). The level of resistance stimulated by the 48-kD antigen was equal to or higher than that stimulated by the most effective crude antigen preparation. The level of resistance stimulated by the 50/55-kD antigen was lower, but still highly significant. A third antigen, the 37-kD antigen, from the body cavity of the infective larva, did not induce resistance at biologically relevant doses. To investigate the mechanism underlying immunologic response to the two identified target antigens of *T. spiralis*, we studied the progression of the parasite's life cycle in control mice and in mice immunized with those antigens.

CFW mice exposed for the first time to *T. spiralis* expel most adult worms from the small intestine 9 to 14 days after infection (*3*). The life-span of adult worms is similar in mice injected with carrier buffer plus complete Freund's adjuvant (Fig. 1). However, mice immunized with the 48-kD or the 50/55-kD antigen expelled most adult worms between days 7 and 11 (Fig. 1). Female adult worms remaining in the intestines of normal mice (*3*) or control mice (Fig. 2) showed diminished fecundity beginning on day 8. Worms from mice immunized with the 48-kD or 50/55-kD antigen showed the same effect as early as day 6 (Fig. 2). Thus, immunization with either target antigen accelerates both the expulsion and antifecundity responses.

Investigators have tested for immune effector mechanisms against newborn larvae by transplanting this stage into control and immunized hosts (*1*). This approach was inappropriate for our purposes because (i) newborn larvae, which are obtained routinely by overnight incubation of gravid female adults (*7*), are

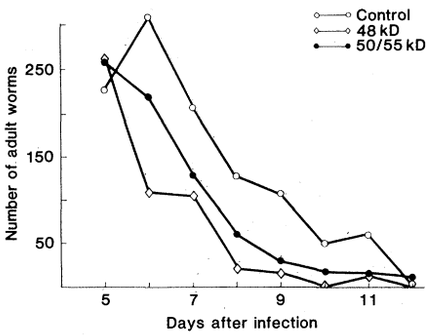


Fig. 1. Numbers of adult worms in immunized mice. Three-month-old male CFW mice (Charles River) were injected three times at weekly intervals with 0.1 ml of emulsified complete Freund's adjuvant (Difco) diluted 1:1 with 0.01*M* sodium phosphate buffer (*p*H 7.2) (control mice) or buffer containing a total of 1 μg of purified antigen protein. One week after the last injection the mice were infected orally with 360 infective stage 1 larvae each. The animals were killed on the indicated days. Their small intestines were removed rapidly, slit lengthwise, rinsed once in distilled water, and incubated individually in 0.85 percent sodium chloride at 37°C. At the 1-hour mark eight female worms per mouse intestine were removed by Pasteur pipette under a dissecting microscope for fecundity assays (see Fig. 2). The remaining worms were counted directly after 4 hours. Data points give the mean number of worms per mouse intestine for five mice on odd-numbered days and for three mice on even-numbered days. Counts for mice immunized with the 48-kD antigen on days 6 through 12 and for the mice immunized with the 50/55-kD antigen on days 7 through 11 were significantly lower than for control mice ($P < 0.05$, Wilcoxon signed-rank test).

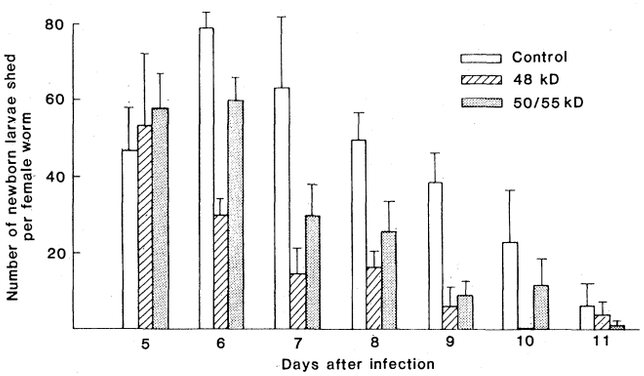


Fig. 2. Fecundity of female worms from immunized mice. On the indicated day, adult female worms, recovered for fecundity assays as described in the legend to Fig. 1, were placed individually in microwells containing 1 ml of Difco medium 199 supplemented with 20 percent calf serum and 1 percent antibiotic-antimycotic solution (Gibco) for 24 hours. After this period newborn larvae were counted directly under a dissecting microscope. Each value is the mean number (+ standard error) of newborn larvae produced per adult female worm per day ($n = 8$ worms in each of five mice on odd-numbered days and in each of three mice on even-numbered days, except $n = 3$ worms in each of five 48-kD–immunized mice on day 11, 5 worms in each of five 50/55-kD–immunized mice on day 11, and 3 worms in each of three 50/55-kD–immunized mice on day 12).

Table 1. Predicted and actual recovery of muscle larvae in control and immunized mice. Immunization was carried out as described in the legend to Fig. 1. Each predicted value is the product of the mean number of adult female worms per mouse ($n = 5$ mice on odd-numbered days and 3 on even-numbered days) and the mean number of larvae per female worm (with $n$ as described in the legend to Fig. 2). Each value for actual recovery is the mean number (± standard error) of muscle larvae recovered per mouse ($n = 7$). The larvae were recovered at 30 days from parallel sets of mice immunized and infected under identical conditions. For complete recovery of larvae, the mice were skinned and eviscerated and the carcasses were minced and digested overnight in 1 percent pepsin and 1 percent concentrated HCl at 37°C. ND, not done because of lack of recoverable adult females.

| Day | Unimmunized mice (controls) | Immunized mice | |
|---|---|---|---|
| | | 48-kD antigen | 50/55-kD antigen |
| | *Predicted recovery* | | |
| 5 | 7,943 | 9,328 | 10,498 |
| 6 | 13,983 | 2,040 | 8,521 |
| 7 | 9,135 | 780 | 2,490 |
| 8 | 3,150 | 69 | 936 |
| 9 | 2,808 | 54 | 232 |
| 10 | 341 | ND | 132 |
| 11 | 210 | 36 | 22 |
| 12 | 68 | ND | 81 |
| Total | 37,638 | 12,307 | 22,911 |
| | *Actual recovery* | | |
| | 43,098 ± 7,597 | 13,655 ± 5,120 | 26,112 ± 5,430 |

Table 2. Antigen concentrations in crude extracts of parasites at different developmental stages. Stage 1 larvae were collected by digestion of minced, infected mouse muscle in 1 percent pepsin and 1 percent concentrated HCl at 37°C for 1 hour. Worms were passed through a 24-gauge sieve and recovered in a 200-gauge sieve. Stage 2, 3, and 4 larvae and adult parasites were collected from infected mouse intestine by the thermal migration method (*6*) at 11, 18, 26, and 170 hours, respectively. Newborn larvae were collected by overnight culture of adult female worms (*7*) and were separated from adult worms by passage through a 200-gauge sieve. The preparation of adult worms was unavoidably contaminated with developing newborn larvae in gravid females. Worms of all stages were washed four times by sedimentation in phosphate-buffered saline (PBS). Values (means ± standard errors for three determinations) represent micrograms of antigen protein per milligram of total protein in crude antigen extracts of worms at specified developmental stages. Extracts were prepared by homogenization of worms in PBS on ice by ten passes through a tissue grinder (Potter-Elvehjem). The homogenized material was centrifuged for 30 minutes at 10,000*g* and the supernatant was diluted with PBS to a concentration of 1 mg/ml. Concentrations of specific antigens in crude extracts were determined by a double antibody sandwich enzyme-linked assay (*5*). Tests of this assay with known dilutions of antigen standards show that it is accurate to within a factor of 2 (*12*).

| Stage | Antigen | | |
|---|---|---|---|
| | 48-kD | 50/55-kD | 37-kD |
| Larval 1 | 4.4 ± 0.3 | 6.0 ± 1.1 | 11.0 ± 4.5 |
| Larval 2 | 3.8 ± 0.8 | 2.1 ± 1.0 | 19.2 ± 6.1 |
| Larval 3 | 1.2 ± 0.6 | 1.7 ± 0.2 | 7.4 ± 2.9 |
| Larval 4 | 0.2 ± 0.3 | 0.3 ± 0.2 | 12.7 ± 1.0 |
| Adult | <0.1 | <0.1 | 34.6 ± 16.5 |
| Newborn | <0.1 | <0.1 | 5.9 ± 1.7 |

antigenically different from true newborns (*8*) and (ii) technical difficulties prevent the introduction of newborn larvae into the host by their natural route, the mesenteric lymph vessels (*9*). However, the data in Figs. 1 and 2 can be used to estimate the efficiency of the migration of newborn larvae. The total number of newborn larvae produced in vivo on each day of infection can be estimated by multiplying the number of newborn larvae produced by adult female worms in 24 hours by the number of females per mouse (Table 1). For both control and immunized mice, the estimated total number of newborn larvae produced during infection agrees closely with the number of muscle larvae recovered from a parallel set of mice that were immunized and infected under identical conditions. This close correspondence between predicted and actual worm counts holds for other sets of data, including the results of two experiments with high doses (50 μg) of purified and crude antigens and published data on a crude antigen (*3*). Under these conditions, most or all newborn larvae that are produced in the intestinal epithelium are able to establish an intramuscular niche. Thus the entire reduction in the number of accumulated muscle larvae in immunized mice can be accounted for by effects against adult worms.

Electron microscopy shows that the contents of stichocyte granules, which include the 48- and 50/55-kD antigens (*5*), are synthesized by the muscle larva during a brief period of its parenteral development and secreted completely during the first 30 hours of intestinal infection (*10*). A quantitative enzyme-linked assay confirms that the 48- and 50/55-kD antigens are depleted during maturation from larval stages 1 to 4 and absent from adult worms or newborn larvae. The 37-kD antigen, on the other hand, is constitutive in all stages (Table 2). These results are consistent with the observation that adult worms are unaffected when transplanted into hosts immunized with antigens of infective stage 1 larvae (*11*). Given that protective antigens are stage-specific (Table 2) (*1, 11*), it is surprising that the immune response we have studied affects the adult worm, a stage that does not express the target antigen. This evidence suggests that the immune response involves the 48- and 50/55-kD antigens of the intestinal larva in such a way that it develops into a defective adult.

Even a light primary infection with *T. spiralis* presents sufficient quantities of 48- and 50/55-kD antigens to stimulate an immune response (*5*). In a secondary infection these antigens are available as targets days earlier than antigens presented by other stages of the parasite. The response to these antigens is sufficient to account entirely for the level of resistance induced by immunization. For these reasons it is probable that the reaction to these antigens forms a major component of the resistance to reinfection.

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**References and Notes**

1. D. Wakelin and D. A. Denham, in Trichinella *and Trichinosis*, W. C. Campbell, Ed. (Plenum, New York, 1983), pp. 265–308.
2. Z. Pawlowski, *ibid.*, pp. 367–398.
3. D. D. Despommier *et al.*, *Parasitology* **74**, 109 (1977).
4. _____ and A. Laccetti, *Exp. Parasitol.* **51**, 279 (1981); *J. Parasitol.* **67**, 332 (1981).
5. D. S. Silberstein and D. D. Despommier, *J. Immunol.* **132**, 898 (1984).
6. D. D. Despommier, *J. Parasitol.* **59**, 933 (1973).
7. D. Dennis *et al.*, *ibid.* **56**, 974 (1970).
8. M. Jungery *et al.*, *Mol. Biochem. Parasitol.* **7**, 101 (1983).
9. A. Basten and P. B. Beeson, *J. Exp. Med.* **131**, 1288 (1970); J. P. Harley and V. Gallichico, *Exp. Parasitol.* **30**, (1970).
10. D. D. Despommier, in *Trichinellosis*, C. W. Kim, Ed. (Intext Educational, New York, 1974), pp. 239–254.
11. R. G. Bell *et al.*, *Exp. Parasitol.* **47**, 140 (1979).
12. D. S. Silberstein, thesis, Columbia University (1984).

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