Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 3155–3158, April 1992
Immunology

Induction of heat shock protein closely correlates with protection against *Toxoplasma gondii* infection

(protective immune response/parasite infection/body defense)

HIDEYUKI NAGASAWA*, MIKIO OKA*, KEN-ICHI MAEDA*, CHAI JIAN-GUO*, HAJIME HISAEDA*, YOSHIHIRO ITO*, ROBERT A. GOOD†, AND KUNISUKE HIMENO*

*Department of Parasitology and Immunology, School of Medicine, The University of Tokushima, Tokushima, Japan; and †Department of Pediatrics, All Children's Hospital, University of South Florida, St. Petersburg, FL 33731

Contributed by Robert A. Good, January 2, 1992

ABSTRACT **Heat shock proteins (HSPs) are evolutionarily highly conserved polypeptides that appear to be produced by many cells to preserve cellular functions under a variety of conditions of stress, including infections. We report that a 65-kDa HSP is present in mouse peritoneal cells that have been infected with a low-virulence (Beverley) strain of *Toxoplasma gondii*, as determined by electroblot assay using a monoclonal antibody specific for microbial HSP65. This HSP is, however, not expressed when infection occurs with the high-virulence RH strain of *T. gondii*. Furthermore, HSP was demonstrable in mice that acquired resistance against infection with a lethal dose of bradyzoites of the Beverley strain or even of an inoculum of a highly virulent strain of *T. gondii* (RH). From these results, it can be suggested that HSPs play an important role in developing effective defenses that include effective immune responses against infection with *Toxoplasma* parasites *in vivo*.**

Exposure of cells to a variety of stressful conditions including elevated temperature, stressful chemical intoxication, or infection leads to the transcription of a highly conserved set of genes and, thus, to the synthesis of a family of polypeptides termed heat shock proteins (HSPs) (1–3). Immunodominant antigens from a wide variety of bacteria and parasites have been identified by sequence homology as belonging to the family of HSPs (4). Recently, HSPs have attracted the attention of immunologists as targets for specific recognition by both antibodies and T cells of the immune system. Among the various HSPs, a 65-kDa mycobacterial HSP has been identified as a target of T cells, including γδ T cells (5–10). This HSP contains a significant sequence similarity and cross-reactivity with antigens from a variety of other microbes. Moreover, monoclonal antibodies (mAbs) against this common bacterial HSP have been used to identify a similar molecule of identical size and specificity that is produced by murine macrophages (7).

Toxoplasma gondii is an obligate intracellular protozoan parasite found throughout the world. We showed earlier that T cells play an important role in protective immunity against infection with *Toxoplasma* parasites (11). However, the protective mechanisms involved in resisting infection with a strain of *T. gondii* of low virulence (Beverley strain) differ greatly from those involved in resisting infection with a highly virulent strain (RH strain). When mice were immunized with *Toxoplasma* cell homogenates 7 days before infection with a lethal dose of Beverley strain bradyzoites (1×10^4), the mice acquired resistance and survived. By contrast, immunization with live *Toxoplasma* organisms appeared to be required for acquisition of resistance to infection with the highly virulent RH strain. This interpretation appears to be supported by observations that immunization with *Toxoplasma* cell homogenates along with complete Freund's adjuvant failed to prevent infection of mice by tachyzoites of the RH strain.

In the present study, we have observed that HSP65 possessing an epitope located between amino acids 172 and 224 of *Mycobacterium bovis* is expressed in host peritoneal exudate cells (PEC) infected with *T. gondii*. The degree of expression of this apparent HSP correlated with protection that occurred in exposed mice, regardless of differences in virulence or strain specificity of this protozoan. The relationship between expression of HSP65 within host cells and the capacity to develop protective immunity against *Toxoplasma* infection has been investigated by using both low-virulence and high-virulence strains of *T. gondii*. Prior expression of HSP65 within PEC strongly correlated with capacity to develop effective immunity against a large dose of the relatively avirulent strain and a lower dose of a more virulent strain of *T. gondii*.

MATERIALS AND METHODS

Animals. Female BALB/c mice purchased from Shizuoka Laboratory Center (Shizuoka, Japan) were used for experiments at 8–10 weeks of age.

Parasites. A low-virulence Beverley strain (12) and a high-virulence RH strain (13) of *T. gondii* were used in these studies. Bradyzoites of the Beverley strain were obtained from cysts isolated from the brains of chronically infected mice. The RH strain of *T. gondii* was maintained by routine passage in mice (14). Bradyzoites and tachyzoites were routinely prepared according to described procedures (14).

Infection and Immunization. Mice were infected with 1×10^4 bradyzoites of Beverley strain or 1×10^4 tachyzoites of RH strain. To induce protective immunity against the low-virulence Beverley strain, mice were injected i.p. with 0.2 mg (total protein) of a *Toxoplasma* cell homogenate and challenged i.p. with 1×10^4 live bradyzoites of Beverley strain 7 days after this immunization as described (11). *Toxoplasma* cell homogenates were prepared from RH strain tachyzoites by three cycles of freeze-thawing. To induce protective immunity against a highly virulent RH strain, mice that had been immunized with *Toxoplasma* cell homogenate and that had survived for 4 weeks after challenge with bradyzoites of the Beverley strain were inoculated i.p. with 1×10^4 tachyzoites of the RH strain.

Immunoblotting. Protein extracts of PEC homogenates derived from mice immunized with *Toxoplasma* cell homogenate or from mice infected with live Beverley or RH strain were mixed with lysate buffer (50 mM Tris·HCl/1% Nonidet P-40/1% SDS/1 μM leupeptin/100 phenylmethylsulfonyl

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HSP, heat shock protein; PEC, peritoneal exudate cells; mAb, monoclonal antibody.

fluoride/1 μM pepstatin A/100 μM EDTA). Protein concentration was determined by the BCA (bicinchoninic acid) protein assay reagent (Pierce). The protein samples were suspended and denatured in sample buffer [0.025 M Tris·HCl, pH 6.8/2% SDS/10% (vol/vol) glycerol/10% (vol/vol) 2-mercaptoethanol/0.002% bromophenol blue] and boiled at 100°C for 3 min. The proteins were separated by SDS/PAGE (12.5% polyacrylamide) at 20 mA for 1 hr. Purified 65-kDa mycobacterial protein was from R. van der Zee (National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands).

The murine mAb IA10, specific for an epitope located between amino acids 172 and 224 of 65-kDa HSP derived from *M. bovis*, was provided by J. DeBruyn (Institute Pasteur de Brabant, Belgium). Gels were electroblotted for 1 hr at 0.8 mA/cm^2 with Sartoblot (Sartorius) onto an Immobilon-P membrane (Millipore). The nitrocellulose blots were rinsed with phosphate-buffered saline (PBS) and blocked for 1 hr in PBS/10% bovine serum albumin (Sigma). The blots were incubated with mAb IA-10 as the first antibody for 1 hr at room temperature and washed in two changes of PBS/0.1% Nonidet P-40 for 30 min. Blots were then incubated for 1 hr with peroxidase-labeled anti-mouse IgG (Tago) and washed in PBS/0.1% Nonidet P-40, followed by a 10-min wash in PBS. Bound antibody was detected by using the Konica immunostaining horseradish peroxidase kit (Konica, Tokyo). The reaction was stopped by washing the nitrocellulose blots in water. The Rainbow protein molecular weight markers (Amersham) were used as the reference molecular size standards.

RESULTS

Expression of HSP on Peritoneal Macrophages of Mice Infected with *T. gondii*. To determine whether 65-kDa HSP could be induced *in vivo* in mice infected with *T. gondii*, mice were infected with two strains differing greatly in virulence—a low-virulence Beverley strain or a high-virulence RH strain. When the mice had been inoculated with a sublethal dose (1 × 10^2) of Beverley strain of *T. gondii* bradyzoites, they suffered from acute symptoms of infection reflected by ruffled hair, hunched posture, and fever on days 10–14 after inoculation. Thereafter the mice regained a healthy appearance and could be shown to have established both immunity and a latent *T. gondii* infection. By contrast, mice infected with 1 × 10^2 tachyzoites of RH strain died within 10 days of an acute infection. As shown in Fig. 1 (lane 3), the expression of HSP as reflected in a electroblot analysis was demonstrable in PEC from mice infected 10 days earlier with the low-virulence Beverley strain of *T. gondii*. In contrast, HSP were not expressed in the macrophages from mice infected with RH strain (lane 4) or in macrophages of uninfected mice (lane 2).

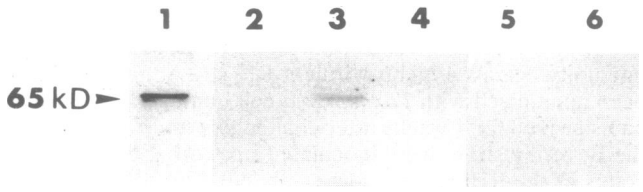

FIG. 1. Expression of 65-kDa (kD) HSP in PEC of mice infected with *T. gondii*. Lanes: 1, HSP65 as standard (10 μg of protein); 2, PEC lysates from normal mouse (10 μg of protein); 3, PEC lysates 10 days after infection of mice with 1 × 10^4 bradyzoites of Beverley strain *T. gondii*; 4, PEC of mice 5 days after infection with 1 × 10^4 tachyzoites of RH strain (10 μg of protein); 5, lysate of tachyzoites of RH strain (5 μg of protein); and 6, lysate of bradyzoites of Beverley strain of *T. gondii* (5 μg of protein) equivalent to ≈1 × 10^6 organisms.

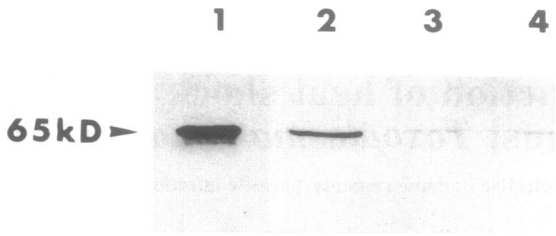

FIG. 2. Expression of 65-kDa (kD) HSP in PEC of mice infected with Beverley strain tachyzoites of *T. gondii* after serial passages. Lanes: 1, recombinant HSP65 as standard (10 μg of protein); 2, PEC lysates from mouse 10 days after infection with 1 × 10^4 bradyzoites of Beverley strain; 3, PEC lysates 10 days after infection with 1 × 10^4 tachyzoites of Beverley strain after a single passage; and 4, PEC after infection with 1 × 10^4 tachyzoites of Beverley strain after passage six times. Loadings were with 10 μg of protein.

As we reported earlier, the pathogenicity of Beverley strain *T. gondii* increases with the serial intraperitoneal passage of the organism in mice, ultimately increasing to become the virulence equivalent of the virulent RH strain (12). Parallel with the decline in capacity to generate resistance, shown in Fig. 2, the expression of HSP65 decreased in the PEC lysate from a high level in the mice infected with bradyzoites (lane 2) to a much lower level in the mice infected with the tachyzoites from even six times-passaged Beverley strain (lane 4).

Induction of HSP on Macrophages of Mice Immunized with *Toxoplasma* Cell Homogenate. When mice had been immunized with *Toxoplasma* cell homogenate 7 days before infection, 90% acquired resistance and survived challenge with an otherwise lethal dose (1 × 10^4) of bradyzoites of the low-virulence Beverley strain. In contrast, all unimmunized control mice injected with the same number of bradyzoites died within 2 weeks after infection (Fig. 3). HSP65 was slightly induced in PEC lysate from mice 7 days after immunization with *Toxoplasma* cell homogenate (Fig. 4, lane 4). This expression of HSP65 became stronger when these mice had been infected with the low-virulence strain of bradyzoites 10 days earlier (Fig. 4, lane 5). HSP65 was not detectable in *Toxoplasma* cell homogenates themselves or in macrophages from naive mice (Fig. 4, lanes 2 and 3, respectively).

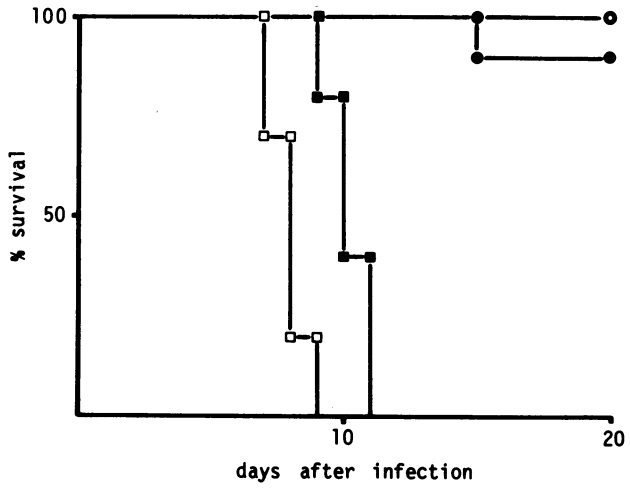

FIG. 3. Effect of immunization on resistance to infection with *Toxoplasma*. Mice were infected with 1 × 10^4 bradyzoites of the Beverley strain 7 days after immunization with (●) or without (■) 0.2 mg of *Toxoplasma* cell homogenates as described. Mice were challenged with 1 × 10^4 tachyzoites of RH strain 5 weeks after immunization and 4 weeks after challenge with bradyzoites from RH strain *T. gondii* with (○) or without (□) immunization.

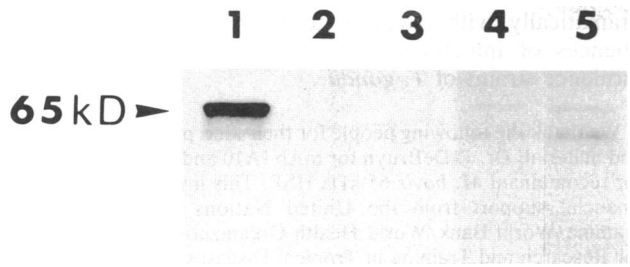

FIG. 4. Electroblot analysis of the expression of HSP65 on macrophages of mice that had acquired resistance against infection with a virulent RH strain. Lanes: 1, recombinant HSP65 as standard (10 μg of protein); 2, PEC lysates from normal mouse; 3, *Toxoplasma* cell homogenates used for immunization (5 μg of protein); 4, PEC lysates from mouse 7 days after immunization with *Toxoplasma* cell homogenate and 10 days after challenge with 1×10^4 bradyzoites of *T. gondii* Beverley strain; and 5, PEC 17 days after immunization with *Toxoplasma* cell homogenates and 10 days after challenge with 1×10^4 bradyzoites of Beverley strain. kD, kDa.

Expression on Macrophages of Mice That Had Acquired Resistance Against Infection with a Virulent RH Strain. We previously showed that mice immunized with *Toxoplasma* cell homogenates (homogenate-immune mice) acquire protective immunity against infection with a low-virulence Beverley strain but do not acquire this immunity against a highly virulent RH strain (11). However, when mice that had survived for 4 weeks after the bradyzoite infection (bradyzoite-immune mice) were inoculated i.p. with a lethal dose (1×10^4) of tachyzoites from the highly virulent RH strain, all survived (Fig. 3). Thus, mice that have recovered from infection with the low-virulence *Toxoplasma* parasite acquired almost complete protective immunity against highly virulent *Toxoplasma* infection. When we examined the expression level of HSP65 according to susceptibility to infection with tachyzoites of a highly virulent RH strain in bradyzoite-immune mice by electroblotting, we found, as shown in Fig. 5, that PEC from bradyzoite-immune mice impressively expressed HSP65 (lane 4). This expression of HSP65 became intense when these mice had been infected with tachyzoites of RH strain 5 days earlier (lane 6). The expression of HSP was not present on macrophages of nonimmune mice or of nonimmune mice that had been infected with a highly virulent RH strain (lanes 2 and 5, respectively).

DISCUSSION

The role of HSPs in infection and immunity is receiving much attention, and it has been postulated that HSP responses to stress during inflammation actually play a role in the body's defense against certain infections (15). HSPs in parasite infections appear, on the one hand, to play important roles in adaptation of microorganisms. For example, they may play roles in differentiation of parasites and in development of infectivity (16, 17). On the other hand, HSPs function as prominent antigenic proteins that can activate the host immune systems (10). Whether the host cells stressed by the invading parasite synthesize HSPs, whether and how host HSPs can affect parasite–host interactions, and whether HSPs generated by host cells or parasites participate in protective immunity and/or in the development of autoimmunities are issues to be resolved.

In the present report, we have demonstrated that the 65-kDa HSP detected and measured by electroblot assay with specific mAb is expressed in peritoneal cells of mice infected with a low-virulence strain of *Toxoplasma* (Beverley) but is not expressed if infected with a high-virulence RH strain. Further, the expression of HSP65 decreased in the PEC

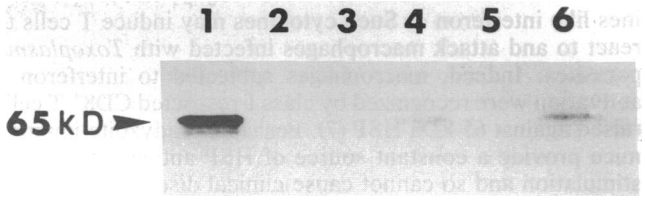

FIG. 5. Electroblot analysis of PEC from immune mice infected with highly virulent strain of *T. gondii*. Lanes: 1, recombinant HSP65 was loaded as standard (10 μg of protein); 2, PEC lysates from normal mouse; 3, lysate from tachyzoites used for infection (5 μg of protein); 4, PEC lysates from mouse 4 weeks after infection with 5×10^4 bradyzoites of Beverley strain and 5 weeks after immunization with *Toxoplasma* cell homogenates; 5, PEC lysates from mouse 5 days after challenge with 1×10^4 tachyzoites of RH strain and 4 weeks after immunization with 1×10^4 bradyzoites of Beverley strain (10 μg of protein); and 6, PEC lysate from mouse 5 days after challenge with 1×10^4 tachyzoites of RH strain that was given 4 weeks after infection with 1×10^4 bradyzoites of Beverley strain and 5 weeks after immunization with *Toxoplasma* cell homogenates. Lanes were loaded with 10 μg of protein, except for lane 3. kD, kDa.

lysate from mice infected with the serially passaged tachyzoites of Beverley strain, which, in turn, increased in pathogenicity to become equivalent to the RH strain with respect to infectivity. These findings suggest that expression of virulence in these parasites correlates with interaction between the host cells and parasites. This correlation occurred despite major differences between the strains of this protozoa. Therefore, it is postulated that the low-virulence strain of *T. gondii* with capacity to persist in host macrophages for prolonged periods may generate production of abundant quantities of host HSP that can then be expressed on the surface of macrophages and, thus, presented effectively to T cells to induce immunity. By contrast, mice infected with a highly virulent strain of *T. gondii* (RH) seem unable to produce HSP on the macrophages they infect.

Mammalian cells may synthesize HSPs in response to infections and/or in response to physiological stimulation. With regard to phagocytes, these cells protect themselves from noxious molecules that they produce, such as highly reactive oxygen metabolites. Indeed, phagocytosis and physiological activators of the oxidative burst induce HSP synthesis in macrophages (15). However, *Toxoplasma* parasites may survive and replicate within macrophages after phagocytosis. The reason for survival of *Toxoplasma* within certain phagocytic cells has been addressed by Wilson *et al.* (18). These investigators showed that survival of tachyzoites within human monocyte-derived macrophages and normal mouse peritoneal macrophages can be attributed to failure of this parasite to stimulate an oxidative burst that normally occurs with phagocytosis of *Candida, Staphylococci* spp, or latex particles.

In our experiments, when mice were immunized with *Toxoplasma* cell homogenates, HSP65 was detectable in PEC from these mice 7 days after immunization but was not detectable from either unimmunized controls or *Toxoplasma* cell homogenates themselves (Fig. 4). Furthermore, mice that acquired resistance against a high-virulence RH strain after the resolution of infection with Beverley strain bradyzoites strongly expressed HSP65 in their PEC (Fig. 5). We previously reported that T cells play a major role in mediating protective immunity against low- and high-virulence strains of *T. gondii* from studies that depleted T cells *in vivo* with a mAb against Thy1.2 (11). Further evidence comes from the finding that interferon γ released from sensitized T cells acts as a major mediator of the host defenses against *T. gondii* infection (19, 20). Taking these results together, one might suggest that the expression of HSPs in mice immunized by *Toxoplasma* cell homogenates may be attributable to cytok-

ines like interferon γ. Such cytokines may induce T cells to react to and attack macrophages infected with *Toxoplasma* parasites. Indeed, macrophages subjected to interferon γ activation were recognized by class I-restricted $CD8^+$ T cells raised against 65-kDa HSP (7). Perhaps, bradyzoite-immune mice provide a constant source of HSP antigens for T cell stimulation and so cannot cause clinical disease. Once activated, macrophages would be expected to rapidly eradicate the highly virulent parasites before clinical disease develops.

HSPs expressed within host cells may also participate in the elimination of pathogens, either by a nonimmunological self-nonself discrimination mechanism, as hypothesized by Forsdyke (21), or as a consequence of processing and presentation of foreign antigens for effective immunity (22). In the course of characterizing T-cell stimulatory antigens of tubercle or leprosy bacilli, T cells with reactivity to the 65-kDa HSP have frequently been identified. In mice immunized with killed *Mycobacterium tuberculosis*, ≈10% of T cells that exhibit reactivity to whole *M. tuberculosis* particles recognize the 65-kDa HSP (23). Moreover, a significant number of healthy individuals possess T cells specific for the mycobacterial 65-kDa HSP (24). Thus, the cellular immune response to the 65-kDa HSP cannot be taken as an indication of immunity to tuberculosis or leprosy, and use of this antigen is inappropriate for diagnosis of these diseases. Still, HSP may contribute to acquired resistance against a variety of intracellular pathogens (25). Because of their high degree of conservation in microbes, the 65-kDa HSP (as well as other HSPs) is likely seen by the immune system quite frequently.

Recently, a subset of $\gamma\delta$ T cells has been shown to recognize HSP65 (8–10). This T cell subset is thought possibly to represent a first line of defense against infection. However, cross-reactivity of HSP65 with other autologous stress proteins of the same HSP family, perhaps triggered by common infectious organisms that share the peptide or that can induce its expression, may result in autoaggressive T cell responses that produce inflammatory tissue injury (8, 26). T cells reacting with the 65-kDa HSP are probably demonstrable in normal individuals, and these HSP-reactive T cells have been primed previously by contact with many different microbes or by exposure to HSP generated in host cells by other stressful conditions. The role of HSPs as selective targets for $\gamma\delta$ T cells remains more speculative than the role of HSPs in other forms of immunologic reactions, particularly nonspecific resistance to parasites. Characterization of effector and regulatory functions of HSPs in other host and microbial systems should provide insights into mechanisms of virulence and protective adaptations that control virulence. Thus, it seems likely that HSPs could assume a critical importance in numerous host–parasite relationships, including resistance of host to otherwise destructively virulent parasites. At any rate, it seems clear from present studies that expression of HSP-65 in peritoneal macrophages correlates dramatically with capacity to inhibit destructive consequences of infection with both low-virulence and high-virulence strains of *T. gondii*.

We thank the following people for their kind provision of reagents and material: Dr. J. DeBruyn for mAb IA10 and Dr. R. van der Zee for recombinant *M. bovis* 65-kDa HSP. This investigation received financial support from the United Nations Development Programme/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases.

1. Lindquist, S. (1986) *Annu. Rev. Biochem.* **55,** 1151–1191.
2. Schlesinger, M. J. (1986) *J. Cell Biol.* **103,** 321–325.
3. Pelham, H. (1988) *Nature (London)* **332,** 776–777.
4. Young, D. B., Lathigra, R., Hendrix, R., Sweetser, D. & Young, R. A. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 4267–4270.
5. van Eden, W., Thole, J. E. R., van der Zee, R., Noordzij, A., van Embden, J. D., Hensen, E. J. & Cohen, I. R. (1988) *Nature (London)* **331,** 171–173.
6. Res, P. C. M., Scharr, C. G., Breedveld, F. C., van Eden, W., van Embden, J. D., Cohen, I. R. & De Vries, R. R. P. (1988) *Lancet* **ii,** 478–480.
7. Koga, T., Wand-Württenberger, A., DeBruyn, J., Munk, M. E., Schoel, B. & Kaufmann, S. H. E. (1989) *Science* **245,** 1112–1115.
8. O'Brien, R. L., Happ, M. P., Dallas, A., Palmer, E., Kubo, R. & Born, W. K. (1989) *Cell* **57,** 667–674.
9. Holoshitz, J., Koning, F., Coligan, J. E., Bruyn, J. D. & Strober, S. (1989) *Nature (London)* **339,** 226–229.
10. Haregewoin, A., Soman, G., Hom, R. C. & Finberg, R. W. (1989) *Nature (London)* **340,** 309–312.
11. Nagasawa, H., Manabe, T., Maekawa, Y., Oka, M. & Himeno, K. (1991) *Microbiol. Immunol.* **35,** 215–222.
12. Ito, Y., Kusunoki, Y., Tsuchimoto, M., Doi, M., Furuya, M., Oka, Y. & Osaki, H. (1976) *Jpn. J. Parasitol.* **25,** 133–140.
13. Sabin, A. B. (1941) *J. Am. Med. Assoc.* **116,** 801–807.
14. Nagasawa, H. (1984) *Jpn. J. Parasitol.* **33,** 265–273.
15. Polla, B. S. & Kantengwa, S. (1991) *Curr. Trop. Microbiol.* **167,** 93–105.
16. Smejkal, R. M., Wolff, R. & Olenick, J. G. (1988) *Exp. Parasitol.* **65,** 1–9.
17. Buchmeier, N. A. & Heffron, F. (1990) *Science* **248,** 730–732.
18. Wilson, C. B., Tsai, V. & Remington, J. S. (1980) *J. Exp. Med.* **151,** 328–346.
19. Gazzinelli, R. T., Hakim, F. T., Hieny, S., Shearer, G. M. & Sher, A. (1991) *J. Immunol.* **146,** 286–292.
20. Subauste, C. S. & Remington, J. S. (1991) *Eur. J. Clin. Microbiol. Infect. Dis.* **10,** 58–67.
21. Forsdyke, D. R. (1985) *J. Theor. Biol.* **115,** 471–473.
22. Vanbuskirk, A., Crump, B. L., Margoliash, E. & Pierce, S. K. (1989) *J. Exp. Med.* **170,** 1799–1809.
23. Kaufmann, S. H. E. (1987) *Microb. Sci.* **4,** 324–328.
24. Munk, M. E., Schoel, B. & Kaufmann, S. H. E. (1988) *Eur. J. Immunol.* **18,** 1835–1838.
25. Kaufmann, S. H. E. (1989) *Curr. Opin. Immunol.* **1,** 431–440.
26. Born, W., Hall, L., Dallas, A., Boymel, J., Shinnick, T., Young, D., Brennan, P. & O'Brien, R. (1990) *Science* **249,** 67–69.