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Author(s): Clotilde K. S. Carlow, M. Kaye Edwards, Eric R. James and Mario Philipp
Source: *The Journal of Parasitology*, Vol. 73, No. 5 (Oct., 1987), pp. 1054-1057
Published by: The American Society of Parasitologists
Stable URL: http://www.jstor.org/stable/3282536
Accessed: 12/04/2013 12:00

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J. Parasit., 73(5), 1987, pp. 1054–1057
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Monoclonal Antibodies to Parasite Antigens: A Rapid Immunization Protocol Requiring Small Numbers of Parasites

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A variety of immunization procedures for the production of murine monoclonal antibodies reacting with protozoan or metazoan parasite antigens have been reported. Immunization protocols include natural courses of infection (Freeman et al., 1980, Nature **284:** 366–368; Ortega-Pierres et al., 1984, Parasite Immunology **6:** 275–284), repeated injections of live organisms (Smith et al., 1982, Parasitology **84:** 83–91; Taylor and Butterworth, 1982, Parasitology **84:** 65–82), of surface-membrane suspensions and extracts (McMahon Pratt and David, 1981, Nature **291:** 581–583; Harn et al., 1985, Molecular and Biochemical Parasitology **16:** 345–354), or a combination of such procedures (Canlas and Piessens, 1984, Journal of Immunology **132:** 3138–3141; Sutanto et al., 1985, Molecular and Biochemical Parasitology **15:** 203–214). In all cases, however, several weeks and relatively large numbers of parasites are necessary to achieve the high levels of circulating antibody which normally indicate that spleen cells are ready for fusion.

In this paper we describe a method with which antibody-secreting hybridomas were produced from fusions performed as early as 3 days after a single injection of 500 infective larvae of the filarial parasite *Brugia malayi* or 500 microfilariae of *Onchocerca cervicalis*. The method, which involves an intrasplenic injection (Spitz et al., 1984, Journal of Immunological Methods **70:** 39–43; Gearing et al., 1985, Journal of Immunological Methods **76:** 337–343) of living worms, is illustrated here by the production of antibodies to surface and secreted antigens.

Female mice (6–8 wk old) of the BALB/cByJ strain (The Jackson Laboratory, Bar Harbor, Maine) were used in all experiments. Living infective larvae and frozen adult worms of *Brugia malayi* were purchased from TRS Laboratories, Athens, Georgia. Microfilariae of *Onchocerca cervicalis* were harvested from samples of skin recovered from the midventral region of freshly slaughtered horses, using a method similar to that described by Bianco et al. (1980, Transactions of the Royal Society of Tropical Medicine and Hygiene **74:** 109–110). The emerged worms were cleared of host debris and cells by gel-filtration on Sephadex G-25 M (Pharmacia, Piscataway, New Jersey), following the general recommendations of Taylor et al. (1984, Transactions of the Royal Society of Tropical Medicine and Hygiene **78:** 707–708). Microfilariae were cryopreserved in liquid nitrogen and thawed immediately before use, as described by Ham et al. (1981, Parasitology **83:** 139–146), but no serum was used either in the freezing or in the thawing/diluting medium. To obtain excretory/secretory (ES) antigens, parasites were incubated at 37 C in serum-free medium (199 or RPMI-1640, GIBCO, Grand Island, New York) containing antibiotics, for as long as viability remained above 95%.

Screening of hybrid supernatants was per-

TABLE I. *Fraction of hybridomas secreting antibodies to* Brugia malayi *surface, somatic, and secreted antigens and* Onchocerca cervicalis *secreted antigens.*

Immunofluorescence	ELISA (range of positive absorbance at 490 nm)*		
		Excretory/secretory products	
Infective larvae *Brugia malayi*	Adult extract *Brugia malayi*	*Brugia malayi*	*Onchocerca cervicalis*
0.8%	2.8% (0.38–1.48)	2.5% (0.51–1.08)	5% (0.10–0.55)

* ELISA reference: positive (A_{490}) = 1.85 (*Brugia malayi* adult), 0.70 (*Brugia malayi* ES), 0.81 (*Onchocerca cervicalis* ES); negative (A_{490}) = 0.11 (*Brugia malayi* adult), 0.39 (*Brugia malayi* ES), 0.04 (*Onchocerca cervicalis* ES).

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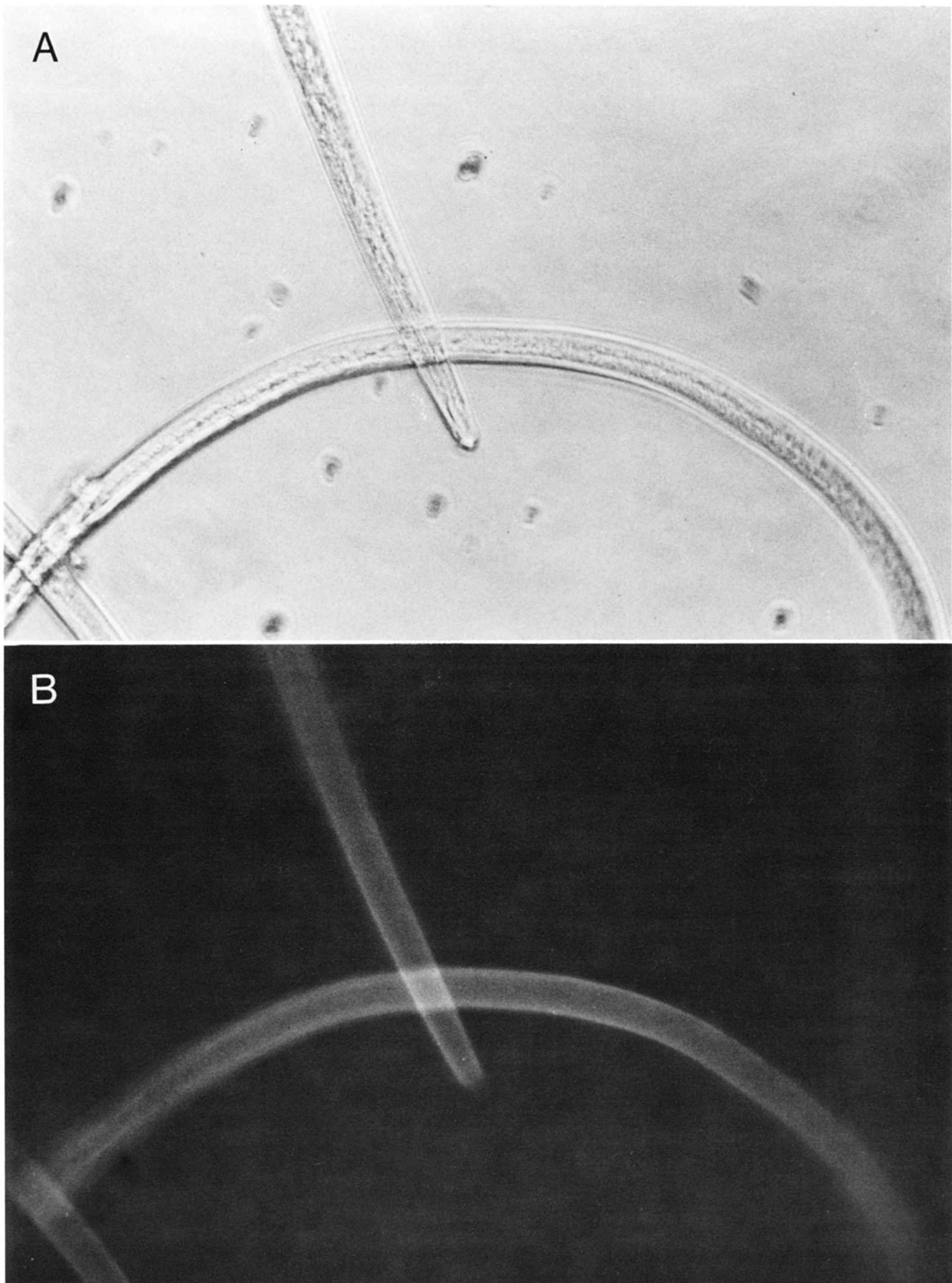

FIGURE 1. *Brugia malayi* larvae incubated with anti-surface monoclonal antibody NEB-D_1E_5 and a FITC-conjugated anti-mouse immunoglobulin antibody, observed under visible light **A**), and UV light **B**). Magnification: ×200. Living third-stage larvae were dispensed suspended in 5 μl of PBS (Dulbecco's phosphate buffered saline, pH 7.00 Gibco, PBS) into flat-bottomed 96-well microtiter plates containing 100 μl undiluted hybridoma culture supernatants to be screened, or a 1:100 dilution of antibody-containing ascites fluid. The incubation was allowed to proceed for 1 hr at 4 C. Following washing in PBS the larvae were incubated for a further 45 min in a 1:20 dilution of a fluorescein isothiocyanate-conjugated goat anti-mouse IgG, IgM, and IgA antibody (Cooper Biomedical, Malvern, Pennsylvania). After washing, the plates were examined under a Nikon Diaphot-TMD fluorescent inverted microscope equipped with a 495 nm main excitation wavelength filter.

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formed both by indirect immunofluorescence (IFAT) on living parasites and by an enzyme-linked immunosorbent assay (ELISA). IFAT involved incubating parasites for 1 hr at 4 C in spent culture supernatants, followed by a 30-min incubation in fluorescein-conjugated goat anti-mouse immunoglobulin antibody (Cooper Biomed, Malvern, Pennsylvania). The antigen preparations used in the ELISA were either detergent–PBS extracts (1.5% of octyl-beta-D-glucopyranoside, Calbiochem, San Diego, California) of *B. malayi* female adult worms (0.5 μg protein/well), ES products of *Brugia malayi* third-stage larvae (1.0 μg protein/well), or *Onchocerca cervicalis* microfilariae (0.5 μg protein/well). The test was peformed using the modified microplate of Voller et al. (1979, *In* Manual of clinical immunology, N. Rose and H. Friedman (eds.), American Society for Microbiology, Washington, D.C., pp. 506–512) with biotinylated goat anti-mouse immunoglobulin antibody (Amersham, Arlington Heights, Illinois) and horseradish peroxidase linked to streptavidin (Amersham).

Intrasplenic injections were performed as described by Spitz et al. (1984, loc. cit.): mice were anesthetized with ethyl ether, the splenic region of the abdomen was shaved, and small incisions were made in the skin (2 cm) and peritoneal membrane (1 cm). The tip of the spleen was exposed and a suspension of live worms in a volume of 50 μl of RPMI-1640 was injected. To ensure a uniform distribution of parasites, the needle (25 ga) was inserted longitudinally, deep into the spleen, and the fluid was dispensed gradually as the needle was withdrawn from the organ (Spitz et al., 1984, loc. cit.). The peritoneal membrane was sutured and the skin was closed with Autoclips (Clay Adams, Parsippany, New Jersey). Splenectomy was performed 3 or 4 days later.

To determine the maximum intrasplenic residence time of injected parasites, 2 groups of 20 mice were given 2,500 microfilariae or 50 third-stage larvae. Spleens were removed from groups of 6 mice on days 1, 2, and 7 after injection, teased apart in medium RPMI-1640 with antibiotics, and inspected after 4 hr incubation at 37 C to detect motile parasites. The remaining 4 spleens were extracted on days 1 and 7 and formalin fixed. Serial sections (5 μm) were obtained from these spleens, stained with hematoxylin/eosin, and examined under the microscope. In a further experiment 2 groups of 3 mice were given 13,000 microfilariae or 300 larvae, respectively. Spleens were inspected both for the presence of motile parasites, or in sections as above on day 1 after injection.

The basic procedure for cell fusion was adapted from that of Fazekas de St. Groth and Scheidegger (1980, Journal of Immunological Methods **35:** 1–21). Hybridomas were screened as above at least twice for antibody production, between days 10 and 14 after fusion. Cells from positive wells were cloned by limiting dilution within 5–10 days of screening, and recloned once thereafter.

To produce monoclonal antibodies to surface and secreted antigens of *B. malayi* infective larvae, spleen cells were fused 3 days after an intrasplenic injection of approximately 500 living larvae. Fusion efficiency assessed 10 days after the fusion was 12 hybridoma colonies/10^6 spleen cells, with an average number of 2.5 colonies/well. Culture supernatants from 6 wells (2.5%) were positive in an ELISA using *B. malayi* ES antigens, and the supernatants of 2 wells (0.8%) contained antibodies that reacted with the surface of living larvae as determined by immunofluorescence (Table I). In addition, an ELISA using an extract of *B. malayi* adult worms yielded 7 positive wells (2.8%) (Table I). All but 1 of the anti-ES antibodies reacted with the adult worm antigen extract. In contrast, antibodies reacting with the larval surface reacted neither with larval ES nor adult worm antigens. Two randomly selected hybridomas secreting anti-ES antibodies, and the 2 showing anti-surface reactivity were cloned as described above. All 4 monoclonal antibodies were found to be IgM (Fig. 1).

Monoclonal antibodies to secreted antigens of microfilariae of *O. cervicalis* were obtained from spleen cells fused 4 days after an intrasplenic injection of about 500 living microfilariae. In this case the fusion efficiency was 40 hybridoma colonies/10^6 spleen cells, with an average number of 5.2 colonies/well. Screening by ELISA with ES antigens from *O. cervicalis* microfilariae yielded a total of 14 positive wells (5%) (Table I). Cells from 4 of these wells were cloned as described. All secreted IgM antibodies.

Antigens on the surface and in the secretions of parasitic helminths have been shown to stimulate protective immunity (Silberstein and Despommier, 1984, Journal of Immunology **132:** 898–904; Smith and Clegg, 1985, Science **227:** 535–538; Urban and Romanowsky, 1985, Experimental Parasitology **60:** 245–254) and to be

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useful in immunodiagnosis of infection (de Savigny et al., 1979, Journal of Clinical Pathology **32:** 284–288; van Knapen et al., 1983, Zeitschrift für Parasitenkunde **69:** 113–118). We decided to produce monoclonal antibodies to such antigens by intrasplenic immunization because of the economy in time and in antigen dose made possible by this method (Spitz et al., 1984, loc. cit.). The fact that spleen cells secreting anti-ES antibodies were available for fusion only 4 days after injecting a total dose of 500 living *Onchocerca* microfilariae confirmed the validity of this approach for use with parasites. In addition, we reasoned that exposure of the spleen to living organisms for very short periods of time could favor the presentation of surface and/or ES antigens over that of internal components of dying worms. This hypothesis, although not yet substantiated, is consistent with our results. On the one hand, direct exposure of the spleen to worm antigens may have been as short as 1 day, for we were unable to recover living larvae, or find parasites in tissue sections of spleens 24 hr after injection. On the other hand, the small proportion of wells (2.8%) containing antibodies reactive with extracts of *B. malayi* adult worms is perhaps suggestive of a limited response to internal antigens. In contrast, a fusion we performed with spleen cells from mice immunized by the more traditional procedure of repeated intraperitoneal injections with living *B. malayi* larvae yielded a higher fraction of culture supernatants (14%) reactive with the same adult worm antigen, even though fusion efficiencies in the 2 experiments were practically the same. However, no antibodies to surface antigens were obtained using the latter procedure.

All antibodies derived by us from intrasplenic immunizations have so far been of the IgM isotype. Using a similar protocol, others have obtained IgG monoclonal antibodies directed against human immunoglobulins, albeit with low frequency (Gearing et al., 1985, loc. cit.). The bias towards the M isotype is a disadvantage of the procedure, because such antibodies are more difficult to purify, and their affinity is often too low. Since parasites did not persist in the spleen for longer than 24 hr, it is unlikely that anti-parasite antibody isotype switching will occur in a large fraction of B cells after a single intrasplenic injection. However, we determined that animals could easily withstand 2 intrasplenic injections administered 7 days apart. Therefore, intrasplenic boosting with small numbers of parasites may be considered to increase the proportion of IgG antibody-producing cells.

Although many species of filarial nematodes can be maintained in the laboratory using rodent hosts, the 2 most prevalent filariae of man, *Wuchereria bancrofti* and *Onchocerca volvulus*, can fully develop only in the Silver Leaf monkey (Palmieri et al., 1980, Journal of Parasitology **66:** 645–651) or the chimpanzee (Duke, 1980, Tropenmedizin und Parasitologie **31:** 41–54). These alternative primate hosts are not commonly available. Monoclonal antibodies to *W. bancrofti* and *O. volvulus* are urgently needed, both for improved immunodiagnosis and as a means to identify protective antigens (Ottesen, 1984, Transactions of the Royal Society of Tropical Medicine and Hygiene **78**[Supplement]: 9–18). The procedure described here should be especially useful when parasite supply is severely limited by restricted host availability. Its brevity, moreover, not only reduces the time invested in generating the final product, but may contribute to the effective presentation of antigens localized at the host–parasite interface.

We thank Ed Kizer, of M and R Packing Company, for assistance in collecting horse skin specimens, and Brad Smith for technical help. Useful comments on this paper by Ted Davis, Ira Schildkraut, Ruth Trimarchi, Patricia Busto, and Ellen Winchell are gratefully acknowledged, together with Marcia Rezza's help in preparing the manuscript.

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