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Acquired Immunity in Experimental Murine Aspergillosis Is Mediated by Macrophages

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A number of studies have substantiated the pivotal role of innate defense mechanisms in protection against invasive aspergillosis. However, experiments demonstrating increased resistance to lethal intravenous (i.v.) infection with *Aspergillus fumigatus* conidia in cortisone-treated or untreated mice preinfected with a sublethal dose of conidia and protection of turkeys inoculated subcutaneously with a killed *A. fumigatus* germling vaccine against subsequent aerosol challenge led us to speculate that acquired immunity may also contribute to host defense against *Aspergillus* infection. Five-week-old male BALB/c mice were inoculated i.v. with 1.0×10^4 viable conidia or saline and challenged i.v. with 1.0×10^6 conidia after 7, 15, or 21 days. No protection against challenge was found after 7 days. However, significant and reproducible protection was observed after 15 and 21 days. Mortality was reduced from 90% in control mice to 53% in preinfected mice 40 days after challenge ($P = 0.0002$). Increased survival was correlated with decreased content of chitin in lungs, liver, and kidneys 4 and 7 days after challenge ($P < 0.05$). Mice were again inoculated with 1.0×10^4 conidia or saline, and after 21 days, 1.0×10^8 or 2.0×10^8 splenocytes were transferred to naive syngeneic recipients; 2.0×10^8 immune splenocytes conferred significant protection ($P = 0.0001$) against i.v. challenge with 1.0×10^6 conidia, and mortality decreased from 83 to 48% 40 days after challenge. Transfer of immune serum offered no protection despite the presence of antibody against a hyphal homogenate of *A. fumigatus*, which was absent in the sera of control mice. Protection by immune splenocytes was maintained after selective depletion of T cells but was abolished after removal of plastic-adherent splenocytes. Adherent cells were characterized as macrophages by using morphological criteria, nonspecific esterase, and MAC-1 monoclonal antibody. Production of hydrogen peroxide by peritoneal and splenic macrophages from preinfected mice was the same as and lower than, respectively, that from uninfected controls. However, phagocytosis of conidia by peritoneal or splenic macrophages from mice preinfected i.v. or intratracheally was significantly increased after 2 and 3 h of coculture compared with that from uninfected animals, whereas in vitro killing of conidia by splenic macrophages was unaltered. Peritoneal or splenic macrophages from control or preinfected mice failed to kill hyphae in vitro. Killing of hyphae by polymorphonuclear leukocytes was not significantly different between mice preinfected i.v. and uninfected controls. Taken together, the results indicate that acquired immunity mediated by activated macrophages can be demonstrated in experimental murine aspergillosis. Although the mechanism is present biologically, its relevance against the invasive hyphal form of *A. fumigatus* is doubtful.

Innate cellular defense mechanisms are of primary importance in host defense against invasive aspergillosis. It appears that the host can call upon pulmonary alveolar macrophages, polymorphonuclear leukocytes (PMNs) and monocytes to form dual graded defense systems against *Aspergillus* species (39). At the pulmonary portal of entry, alveolar macrophages prevent germination and kill conidia in vitro and rapidly eradicate conidia in vivo, after inhalation challenge, even in neutropenic and athymic mice. On the other hand, conidia which evade killing by pulmonary alveolar macrophages germinate to form hyphae which are killed by PMNs and monocytes. Myelosuppression renders mice susceptible only when the first line of defense is overpowered by high challenge doses or by cortisone suppression of the conidiacidal activity of macrophages. This study (39) and others (19, 27, 40, 45) suggested that the alveolar macrophage is directed against conidia and that PMNs and monocytes are directed against hyphae (14–17, 25, 38). Pulmonary alveolar macrophages kill or inhibit germination of *Aspergillus* conidia more efficiently than peritoneal macrophages, which suggests that the site of differentiation of monocytes to mature tissue macrophages influences these properties (40). *Aspergillus fumigatus* conidia are resistant to killing by PMNs because of their relative resistance to neutrophil oxidants as well as suboptimal stimulation of the PMN respiratory burst. However, when the conidia are incubated in broth until they become metabolically active and swell, but do not yet germinate, their sensitivity to PMN oxidants increases, they stimulate a greater respiratory burst, and they generate more chemotactic factors than metabolically dormant, resting conidia (25, 27).

Humoral immunity does not appear to contribute to host defense in invasive aspergillosis (39). Even in the absence of serum, PMNs attach to hyphae and degranulate; the morphology of hyphae appears to be altered (17). Likewise, addition of serum that contains *Aspergillus* precipitins does not affect hyphal damage by monocytes (16).

Although innate immunity by phagocytes is critical in initial defense against invasive aspergillosis, acquired immunity may also play a role later in the course of disease. The following observations suggest its existence. (i) There was increased resistance to lethal intravenous (i.v.) infection with *A. fumigatus* conidia in cortisone-treated (24) or un-

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treated (42) mice preinfected with a sublethal dose of conidia. (ii) Subcutaneous inoculation of turkeys with a killed *A. fumigatus* germling vaccine protected the animals against subsequent aerosol challenge (36). (iii) Suzuki and Hayashi reported that purified galactomannan cell wall polysaccharide antigen from *A. fumigatus* containing negligible quantities of nitrogen was capable of inducing positive delayed skin responses and macrophage migration inhibition in sensitized guinea pigs (44). (iv) Studies by Williams et al. (46) suggested a role for immune glass-adherent cells and T lymphocytes. However, peritoneal macrophages were used, and killing of *A. fumigatus* conidia depends on the anatomical source of the macrophage (40). Furthermore, *nu/nu* mice were not more susceptible to *A. fumigatus* than *nu/+* animals, suggesting that T lymphocytes are unimportant in defense; other experiments demonstrated that *nu/+* mice were protected by immunization in comparison with unimmunized *nu/+* animals, while immunized *nu/nu* mice were not protected in comparison with *nu/nu* controls, implying a role for the T lymphocyte. (v) Patients with chronic granulomatous disease who survived *Aspergillus* infection had significantly elevated lymphocyte blastogenic responsiveness (21). (vi) Antilymphocyte serum increased mortality in experimental aspergillosis (11), although the preparation used may have contained antibodies to leukocytes other than lymphocytes. Facts which argue against acquired immunity are the following. (i) *nu/nu* mice were no more susceptible than their heterozygous littermates to i.v., intraperitoneal, or inhalation infection with *A. fumigatus* (46), although a trend to increased susceptibility was seen in *nu/nu* mice. (ii) Athymic mice and heterozygous littermates eliminated conidia at the same rate from the liver, brain, spleen, and lungs (39). (iii) The inflammatory response to *A. fumigatus* in tissue consists of PMNs, without the participation of lymphocytes. (iv) There is an absence of clinical data suggesting a connection between invasive aspergillosis and deficient T-cell-mediated immunity (37).

To establish the existence of acquired immunity in invasive aspergillosis, we first demonstrated that mice preinfected with a sublethal dose of *A. fumigatus* conidia are protected against subsequent lethal challenge. We then determined that immune splenocytes, but not immune serum, transferred protection to naive syngeneic recipients. Protection was maintained after depletion of T lymphocytes but was abolished after removal of plastic-adherent splenocytes made up predominantly of activated macrophages. Finally, in vitro studies suggested that enhanced clearance of the i.v. challenge dose of conidia by activated macrophages could account for the observed protection.

(This work was presented in part at the 92nd General Meeting of the American Society for Microbiology, New Orleans, La., 26 to 30 May 1992 [28a].)

MATERIALS AND METHODS

Mice. Male 5-week-old BALB/c mice (17 to 20 g), obtained from Canadian Breeding Laboratories, Inc. (St-Constant, Quebec, Canada), were used to establish a breeding colony. All mice were maintained under conventional conditions and fed mouse chow (Prolab Animal Diet; Agway Inc., Syracuse, N.Y.) and water ad libitum. Prospective surveillance of the animals demonstrated the absence of specific pathogenic viruses, bacteria, and parasites throughout the course of the experiments.

Microorganism and culture conditions. *A. fumigatus* 2085, originally obtained from the London School of Hygiene and Tropical Medicine, was grown on Sabouraud dextrose agar (GIBCO Laboratories, Madison, Wis.) supplemented with chloramphenicol for 4 days at 22°C.

Preparation of inocula. Conidia were harvested by washing the slant culture with 5 ml of 0.9% NaCl and shaking it vigorously. Coarse debris was allowed to settle by gravity, and the suspension was decanted into 15-ml plastic conical tubes. A monodisperse conidial suspension was then obtained by briefly shaking the tubes with 3-mm glass beads (Fisher Scientific Co., Fair Lawn, N.J.). Conidia were counted in a hemacytometer, and the suspension was diluted to yield 5.0×10^4 or 5.0×10^6 conidia per ml. The viability of the conidia was >99%, determined by serially diluting and plating out the inoculum on Sabouraud dextrose agar.

Animal inoculations. Mice were pretreated with saline or a sublethal dose of 1.0×10^4 viable conidia in 0.2 ml of saline injected i.v. via the lateral tail vein. In preliminary experiments, no deaths resulted from the sublethal infection after 1 year. All mice were challenged i.v. 7, 15, or 21 days after sublethal infection with a lethal dose of 1.0×10^6 viable conidia, and deaths were recorded daily for 40 days after challenge.

Other groups of 5-week-old mice were inoculated intratracheally (13) with viable conidia suspended in 25 μl of saline or saline alone. In preliminary experiments, intratracheal inoculation of graded inocula produced the following mortalities after 40 days: 2.1×10^7 and 2.5×10^7, 0%; 3.0×10^7, 70%; 6.0×10^7, 84%; 1.2×10^8, 93%. An inoculum of 5.0×10^7 conidia was selected to assess the effect of intratracheal inoculation on phagocytosis and killing of conidia and hyphae by peritoneal or splenic macrophages in vitro in survivors 21 days after inoculation.

Adoptive transfer. Adoptive transfer was carried out 21 days after inoculation with saline or a sublethal dose of 1.0×10^4 viable conidia. Spleens were removed aseptically from donor mice for transfer of spleen cells. Spleens were homogenized in Hanks' balanced salt solution (HBSS; GIBCO), pooled, washed once in cold HBSS (4°C), and treated with 10 ml of Tris-buffered ammonium chloride to lyse erythrocytes. The cells were washed once in cold HBSS, counted, and resuspended in an appropriate volume of HBSS so that 0.5 ml contained 1.0×10^8 or 2.0×10^8 splenocytes. Splenocytes from preinfected or control donors were injected i.v. into 8-week-old recipient BALB/c mice. The viability of splenocytes was >95% as determined by trypan blue exclusion.

Blood was also obtained 21 days after inoculation with saline or a sublethal dose of 1.0×10^4 viable conidia. Sera were pooled, and 0.5 ml was injected i.v. into 8-week-old recipient BALB/c mice.

All recipient mice were challenged i.v. with a lethal dose of 1.0×10^6 viable conidia 24 h after transfer of serum or splenocytes. Deaths were recorded daily for 40 days after challenge.

Depletion of T lymphocytes. Total spleen cell populations (1.0×10^7 cells per ml) were suspended in minimal essential medium (GIBCO) containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Flow Laboratories, Inc., McLean, Va.) and 0.3% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). Splenocytes were depleted of T lymphocytes by incubation in a 1:20 dilution of rabbit anti-mouse T-cell serum (associated Thy-1 antiserum; Cedarlane Laboratories, Hornby, Ontario, Canada) for 60 min at 4°C in a 50-ml plastic conical tube and then by the addition of a 1:12 dilution of Low-Tox-M rabbit complement (Cedarlane) for another 60 min at 37°C. As controls, the cells

were treated with antiserum or complement alone. After treatment, naive recipient 8-week-old BALB/c mice were divided into four groups and received i.v. 2.0×10^8 control spleen cells, 2.0×10^8 sensitized spleen cells, 2.0×10^8 control spleen cells depleted of T lymphocytes, or 2.0×10^8 sensitized spleen cells depleted of T lymphocytes. All recipient mice were challenged i.v. with a lethal dose of 1.0×10^6 viable conidia, 24 h after transfer of splenocytes.

Depletion of adherent cells. Total splenocytes were resuspended in minimal essential medium containing 20% heat-inactivated fetal bovine serum (Flow), 10 mM HEPES, 20 μM L-glutamine (GIBCO), and 50 μM 2-mercaptoethanol (Sigma).

Adherent macrophage monolayers were obtained by plating the spleen cell populations (1.0×10^7 cells per ml) in 175-cm^2 disposable tissue culture flasks (Falcon; Becton Dickinson Labware, Lincoln Park, N.J.) at 2.0×10^8 cells per flask for 60 min at 37°C in 5% CO_2, 95% air, and a humidified atmosphere. Each flask was then washed with cold (4°C) HBSS to remove nonadherent cells. The cells were washed once in cold HBSS, counted, and resuspended in an appropriate volume so that 0.5 ml contained 2.0×10^8 splenocytes. After depletion, naive recipient 8-week-old BALB/c mice were divided into four groups and received i.v. 2.0×10^8 control spleen cells, 2.0×10^8 sensitized spleen cells, 2.0×10^8 control spleen cells depleted of adherent cells, or 2.0×10^8 sensitized spleen cells depleted of adherent cells. All recipient mice were challenged i.v. with a lethal dose of 1.0×10^6 viable conidia 24 h after transfer of splenocytes.

Estimation of spleen cell populations before and after depletions. The proportion of splenocytes composed of T cells, B cells, and macrophages was estimated for total spleen cells, T-cell-depleted splenocytes, adherent splenocytes, and adherent cell-depleted splenocytes. T cells, B cells, and macrophages were quantitated by indirect immunofluorescence by using associated Thy-1 antiserum, anti-mouse immunoglobulin M and immunoglobulin G antiserum (Jackson Immunoresearch, Inc., West Grove, Pa.), and monoclonal antibody to murine or human Mac-1 antigen (Hybritech, Inc., San Diego, Calif.), respectively. Normal mouse thymus and peritoneal macrophages were used as positive controls for Thy-1 antiserum and Mac-1 monoclonal antibody. Macrophages were also estimated by staining for nonspecific esterase.

Chitin assay. The chitin assay was adapted from the method of Lehmann and White (23). Liver, lung, and kidney samples from control, preinfected, and challenged mice were weighed and homogenized in 5 ml of 0.9% NaCl. Homogenates were transferred to 15-ml graduated Pyrex centrifuge tubes and centrifuged at $1,500 \times g$ for 10 min. The supernatant was removed, and the pellet was resuspended in 4 ml of sodium lauryl sulfate (3% [wt/vol]; Fisher) and heated at 100°C for 15 min. After cooling, the tubes were recentrifuged and the supernatant was removed. The pellet was washed once with distilled water, resuspended in 3 ml of KOH (120% [wt/vol]) solution, and heated at 130°C for 1 h. After cooling, the alkaline solution was mixed with 8 ml of ice-cold ethanol (75% [vol/vol]) and the tubes were shaken until the alkaline solution and ethanol formed a single phase. The tubes were kept at 4°C for 15 min, and 0.3 ml of Celite suspension (the supernatant left when 1 g of Celite 545 [Fisher] was mixed with 75% ethanol and left to stand for 2 min) was then added. The tubes were centrifuged ($1,500 \times g$, 10 min, 4°C), the supernatant was discarded, and the pellet was washed once with 10 ml of ice-cold ethanol (40% [vol/vol]) and twice with cold (4°C) distilled water (10 ml). The pellet containing chitosan was stored at 4°C in a 15-ml conical tube.

To the pellet of chitosan, 0.5 ml of $NaNO_2$ (5% [wt/vol]) and 0.5 ml of $KHSO_4$ (5% [wt/vol]) were added. All tubes were mixed three times during a 15-min period and then centrifuged ($1,500 \times g$, 10 min, 4°C). Volumes of 0.6 ml of supernatant were taken, 0.2 ml of ammonium sulfamate ($NH_4SO_3NH_2$, 12.5% [wt/vol]) was added, and the tubes were mixed vigorously each minute for 5 min. Freshly made MBTH (0.2-ml volume; 50 mg of 3-methyl-benzo-2-thiazolone hydrazone HCl monohydrate [Eastman Kodak Co., Rochester, N.Y.] in 10 ml of water) was added, and the tubes were heated in a boiling water bath for 3 min and cooled. At that time, 0.2 ml of $FeCl_3 \cdot 6H_2O$ (0.83% [wt/vol]) was added. After the tubes had been left standing for 30 min, the optical density at 650 nm was read in a Bausch & Lomb Spectronic 20 spectrophotometer.

Dot immunoassay. The presence of antibodies to *A. fumigatus* in immune serum used for passive transfer was verified by dot immunoassay. Nitrocellulose strips were incubated for 10 min in Tris-buffered saline (0.01 M Tris-HCl, 0.15 M NaCl [pH 7.4]) and dried at 37°C. Two microliters of an *A. fumigatus* hyphal homogenate (43) was deposited on the strips, and remaining sites were blocked by successive incubations in Tris-buffered saline supplemented with 0.05% Tween 20 and Tris-buffered saline containing normal goat serum. Nitrocellulose strips were incubated for 45 min at room temperature in pooled serum from control or preinfected mice, diluted 1:20 in Tris-buffered saline containing Tween 20. After the strips were washed, biotinylated goat anti-mouse immunoglobulin G (Vector Laboratories, Burlingame, Calif.) was added and the strips were incubated for 1 h at room temperature. After being washed, the strips were incubated in the Vectastain ABC reagent (a preformed complex between avidin and biotinylated horseradish peroxidase; Vector) for 1 h. The blots were washed, and chromogenic substrate, a solution containing 4-chloro-1-naphthol diluted in Tris-buffered saline, was added. Development was stopped by washing the strips several times in distilled water.

Production of hydrogen peroxide by peritoneal macrophages. Peritoneal cells were harvested by washing the peritoneal cavity of five control or preinfected mice with 10 ml of cold calcium- and magnesium-free HBSS with gentle massage to dislodge any loosely adherent cells. Lavaged cells were centrifuged ($300 \times g$, 10 min) and pooled. Pooled cells were washed once with RPMI 1640 tissue culture medium (GIBCO) containing 15% fetal bovine serum, 4 mM L-glutamine, 20 mM HEPES, 50 μM 2-mercaptoethanol, and antibiotic mixture (100 U of penicillin and 100 μg of streptomycin [GIBCO] per ml). The cells were counted and suspended at 2.0×10^6 cells per ml of culture medium. Macrophage monolayers were established by seeding 2.0×10^6 cells in 35-mm plastic petri dishes (Falcon 3001), incubating them for 36 h at 37°C in 5% CO_2-95% air, and washing them with warm HBSS to remove nonadherent cells. Production of H_2O_2 was measured by the method of Pick and Keisari (34), with phenol red as the oxidizable substrate. Petri dishes containing macrophages received 1 ml of a solution containing phenol red (0.56 mM; Sigma), horseradish peroxidase (20 U/ml; Sigma), and 5.5 mM dextrose in balanced salt solution with or without 100 ng of phorbol 12-myristate 13-acetate (PMA; Sigma) per ml. A reagent blank was obtained by dispensing the same solution into empty petri dishes. After the petri dishes were incubated for 3 h at 37°C, the reaction was stopped with the addition of 10

μl of 1 N NaOH and the A_{610} was measured with a Pye Unicam model SP8-100 spectrophotometer. Concentrations of H_2O_2 were calculated with respect to a standard curve prepared from known concentrations of fresh reagent hydrogen peroxide. The amount of cell protein was determined by covering the monolayers with 1 ml of 0.5 N NaOH, allowing them to stand overnight at 37°C, and performing the protein assay on the digest by the method of Lowry et al. (28). Results were expressed as nanomoles of H_2O_2 per milligram of macrophage protein per hour of incubation (34).

Phagocytosis of conidia by peritoneal macrophages. Peritoneal cells from two control or preinfected mice were harvested as described for peroxide production and pooled. Pooled cells were washed once in supplemented RPMI 1640 medium, counted, and suspended at 1.0×10^6 cells per ml of RPMI 1640 medium. Macrophage monolayers were formed by incubating 1 ml of cell suspension per well of a 24-well tissue culture plate (Flow), containing a 15-mm-diameter round coverslip, at 37°C in 5% CO_2-95% air for 18 h. After incubation, nonadherent cells were aspirated and the monolayers were challenged with 1 ml of *A. fumigatus* conidia (5.0×10^5 conidia per ml of supplemented RPMI 1640 medium, opsonized with 5% autologous serum from control or preinfected mice). Incubation of cocultures was continued with shaking for 1, 2, 3, or 4 h at 37°C in 5% CO_2-95% air, and the wells were aspirated and rinsed once with RPMI 1640 medium. The macrophage monolayers were fixed for 5 min with methanol, and the coverslips were stained by using the Gomori-Grocott methenamine silver procedure. The proportion of adherent cells containing conidia as well as the number of conidia phagocytized per cell was determined in the first 100 randomly examined cells.

Killing of conidia by peritoneal macrophages. Peritoneal cells from two control or preinfected mice were obtained as described for peroxide production, pooled, washed once in supplemented RPMI 1640 medium, and suspended at 5.0×10^6 cells per ml of RPMI 1640 medium. Macrophage monolayers were established by incubating 1 ml of cell suspension per well of a 24-well tissue culture plate containing 1 ml of supplemented RPMI 1640 medium per well at 37°C for 18 h. After incubation and aspiration of nonadherent cells, the monolayers were challenged with 1 ml of opsonized *A. fumigatus* conidia (2.5×10^6 conidia per ml of supplemented RPMI 1640 medium) and incubated for 4.5 h with shaking. Control wells contained conidia alone. After incubation, well supernatants were transferred to 50-ml tubes containing 8 ml of distilled water. The cell monolayers were lysed by adding 1 ml of distilled water to each well and then by freezing and thawing. The wells were washed repeatedly with distilled water, and well washings were pooled with their respective supernatants to avoid a potential skew of viability resulting from ingestion of multiple conidia. Microscopic examination of washed wells showed a complete removal of macrophages. To determine the number of CFU per well, 100 μl of a 10-fold dilution was plated on a Sabouraud-chloramphenicol plate, and colonies were counted after 24 h of incubation at 37°C. Killing of conidia by peritoneal macrophages was calculated by comparing CFU in wells with or without macrophages.

Killing of hyphae by peritoneal macrophages. Peritoneal cells from two control or preinfected mice were obtained as described for peroxide production, pooled, washed once in supplemented RPMI 1640 medium, and suspended at 4.0×10^6 cells per ml of RPMI 1640 medium. Macrophage monolayers were prepared by incubating 100 μl of cell suspension per well of a flat-bottom 96-well tissue culture plate (Nunclon, Roskilde, Denmark) containing 100 μl of supplemented RPMI 1640 medium per well at 37°C for 18 h. Hyphae with an average length of 40 μm were obtained by incubating 3.0×10^6 conidia per ml in a 10-ml Erlenmeyer flask containing 5 ml of minimal essential medium supplemented with 200 mM glutamine, 1% vitamins, 0.1 mM nonessential amino acids, penicillin (100 U/ml), and streptomycin (100 μg/ml) for 18 h at 25°C. After incubation and aspiration of nonadherent cells, the monolayers were challenged with 4.0×10^4 hyphae and incubated for 4 h at 37°C with mild agitation. The culture plates were centrifuged, the supernatants were removed, and 200 μl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma), at a concentration of 0.75 mg/ml in 100 mM phosphate buffer (pH 7.2) containing 2 mg of NADH (Sigma) per ml, was added to each well. Incubation was continued for 2.5 h at 37°C in the dark. Hyphae killed with 10% formaldehyde served as negative controls. The plates were centrifuged, the supernatants were aspirated, and 200 μl of isotonic saline was added to each well. The viability of the hyphae was determined by counting the proportion stained purple by MTT-formazan among the first 100 hyphae observed with an inverted microscope (26).

Phagocytosis of conidia by splenic macrophages. Spleens from two control or preinfected mice were homogenized in calcium- and magnesium-free HBSS, pooled, washed once in cold HBSS, and treated with Tris-buffered ammonium chloride to lyse erythrocytes. The cells were washed three times in cold HBSS, resuspended in supplemented RPMI 1640 medium, and counted. Macrophage monolayers were formed by incubating 3.0×10^7 cells in 60-mm plastic petri dishes (Corning Glass Works, Corning, N.Y.) for 3 h at 37°C. The dishes were washed twice with phosphate-buffered saline (PBS), and the supernatants were discarded. After a second seeding of 3.0×10^7 splenocytes, the dishes were incubated at 37°C for 18 h. Conidia were labeled by incubating 1.0×10^8 conidia per ml of 0.5 M carbonate buffer (pH 9.5) with 3 mg of fluorescein isothiocyanate (Sigma) per ml for 18 h at 4°C with mild agitation. The conidia remained viable after labeling. The conidia were washed twice with 0.9% NaCl, passed three times through a 5-μm-pore-size filter (Thompson BSH, Town of Mount Royal, Quebec, Canada) and counted. After incubation, nonadherent cells were aspirated from the petri dishes, and the monolayers were challenged with 1.0×10^7 conidia. Incubation of cocultures was continued with intermittent shaking for 1, 2, or 3 h at 37°C. The wells were aspirated and washed three times with PBS, and the supernatants were discarded. Adherent cells were harvested by treating the dishes with 0.5 ml of 0.05% trypsin–0.02% EDTA (Flow) for 4 min at 37°C, and the reaction was stopped by adding RPMI 1640 medium supplemented with 15% fetal bovine serum. The cells were washed three times with PBS and fixed with 1% paraformaldehyde. Portions of the cells were examined in a Philips EM 300 electron microscope, as previously described (43), and the remaining cells were analyzed by flow cytometry. The flow analysis was performed with a FACStar flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) equipped with a water-cooled 2-W argon ion laser operating at 488 nm and a 200-mW light output. Multiparametric data were acquired for 10,000 events and analyzed by using Consort 30 software. The flow system was equipped with a 75-μm nozzle tip, and the analysis was performed at a flow rate of 500 events per s. Green (fluorescein isothiocyanate) fluorescence was collected in linear or log scale by using a 530/30 nm filter. Data were analyzed in monoparametric histograms.

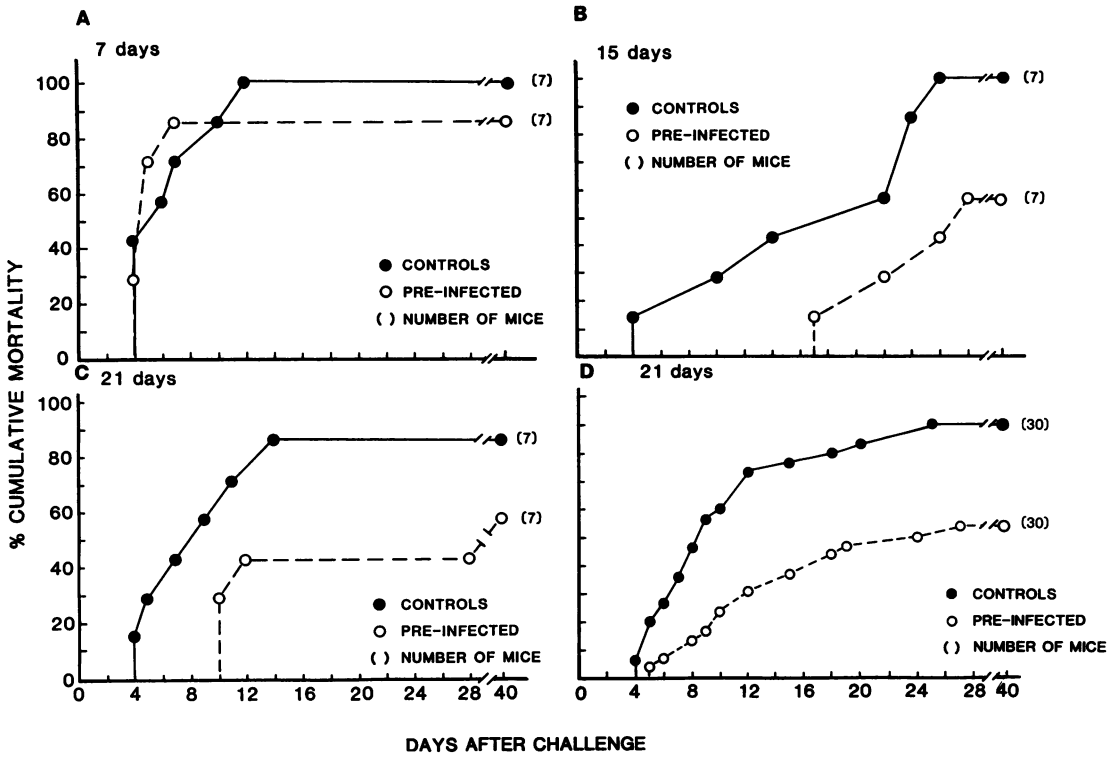

FIG. 1. Cumulative mortality of 5-week-old male BALB/c mice inoculated i.v. with saline or a sublethal dose (10^4) of viable conidia of *A. fumigatus* and challenged after 7 (A), 15 (B), or 21 (C) days with 10^6 conidia. Protection after 21 days (C) was confirmed in a second experiment (D) by using a larger number of animals.

Killing of conidia by splenic macrophages. Fluorescein-labeled conidia and splenic macrophages were cocultured as described for phagocytosis. After 1 h, the petri dishes were washed four times with PBS to remove extracellular conidia. A portion of the dishes was reserved, and incubation of the remaining dishes was continued for a further 5 h after adding supplemented RPMI 1640 medium. The cell monolayers in all dishes were lysed by adding distilled water and then freezing and thawing. The contents of the dishes were transferred to 50-ml tubes. The dishes were washed repeatedly with distilled water, and the washings were pooled. The tubes were centrifuged at 1,200 × *g* for 15 min, and the pellets were transferred to flow cytometry tubes (Falcon 2054). At that time, 200 μl of a 100-μg/ml solution of propidium iodide (Sigma) was added to each tube, and incubation was continued for 30 min at 4°C. After 3 ml of PBS was added, the tubes were centrifuged at 1,200 × *g* for 10 min, and the double-labeled conidia were analyzed by flow cytometry. The flow analysis was performed as described for phagocytosis of conidia, with the additional collection of red (propidium iodide) fluorescence by using a 630/42 nm filter. Green (fluorescein isothiocyanate) and red fluorescence were collected in log and linear scales, respectively, and the data were analyzed in contour graphs. Percent killing of conidia after incubation in 10% formaldehyde at 22°C was comparable when estimated by CFU and binding of propidium iodide (0 min, 0 and 6.5%; 30 min, 99.9 and 92.2%; 60 min, 100.0 and 95.1%, respectively).

Killing of hyphae by splenic macrophages. Splenic macrophage monolayers were prepared as described for phagocytosis of conidia. After incubation and aspiration of nonadherent cells, the monolayers were challenged with 4.0×10^4 hyphae and incubated for 4 h at 37°C with mild agitation. Viability of the hyphae was determined by the MTT method (26).

Production of hydrogen peroxide by splenic macrophages. Splenic macrophage monolayers were prepared as described for phagocytosis of conidia. After incubation, the petri dishes were washed twice with PBS and the supernatants were discarded. Production of hydrogen peroxide was quantitated by flow cytometry by using a modification of the method of Bass et al. (1) because the numbers of adherent cells were insufficient for measurement by the method of Pick and Keisari (34). Two milliliters of supplemented RPMI 1640 medium containing 5 mM sodium azide (Baker Chemical Co., Philipsburg, N.J.) and 50 μM 2',7'-dichlorofluorescin diacetate (Eastman Kodak) was added to each dish, and incubation was continued for 30 min at 37°C with shaking. The dishes were washed with PBS, and 2 ml of supplemented RPMI 1640 medium containing 5 mM sodium azide and 100 ng of PMA per ml was added. After incubation for 40 min at 37°C with shaking, the dishes were washed with PBS and the adherent cells were harvested by treating the dishes with 0.5 ml of 0.05% trypsin–0.02% EDTA for 4 min at 37°C. The contents of the dishes and two washings with PBS were transferred to 50-ml tubes, and the tubes were centrifuged at 600 × *g* for 10 min. The pellets were transferred to flow cytometry tubes (Falcon 2054) and centrifuged at 600 × *g* for 10 min, and the pellet, suspended in a volume of 200 μl, was analyzed by flow cytometry. Green fluorescence was collected as described for phagocytosis of conidia, and the data were analyzed in single-parameter histograms. Autofluorescence of macrophages treated with neither 2',7'-dichlorofluorescin diacetate nor PMA was excluded by use of

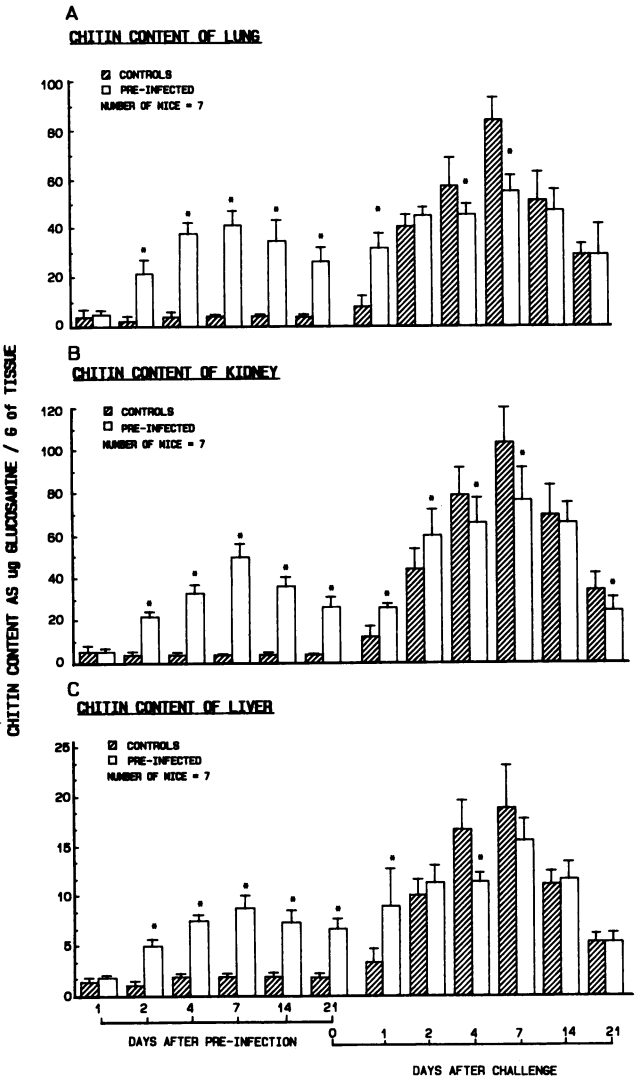

FIG. 2. Chitin content of the lungs, kidneys, and livers of 5-week-old male BALB/c mice inoculated i.v. with saline or a sublethal dose (10^4) of viable conidia of *A. fumigatus* and challenged after 21 days with 10^6 conidia. Vertical bars represent standard deviations. *, $P < 0.05$ for preinfected mice compared with controls.

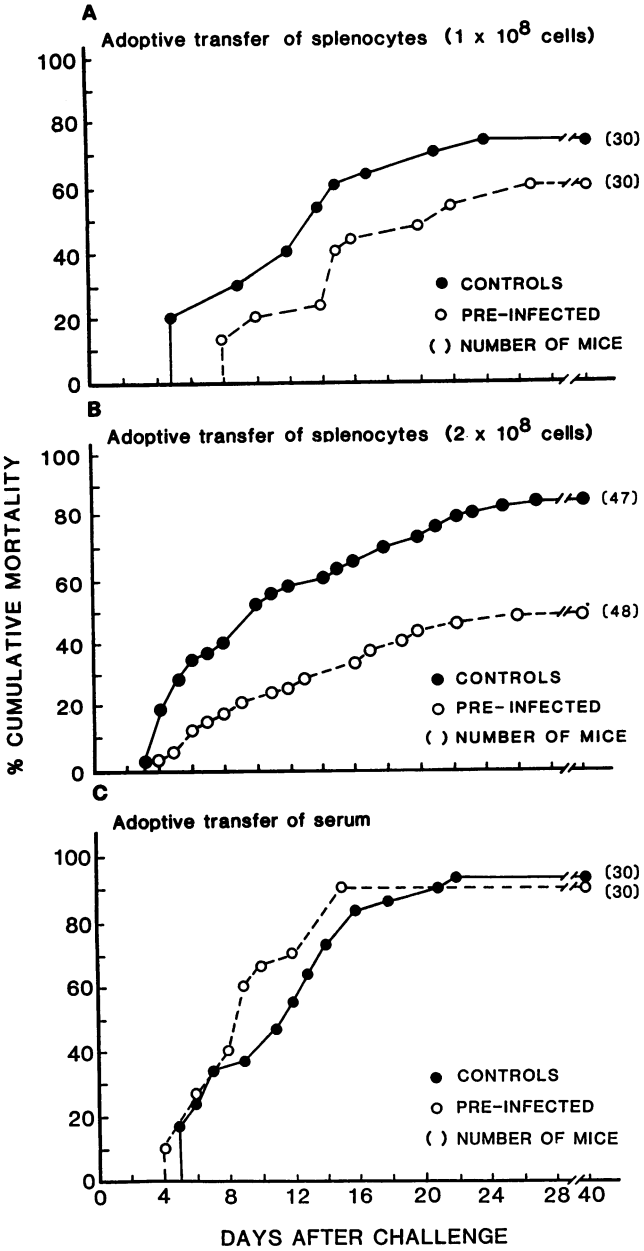

FIG. 3. Effect of transfer of splenocytes or immune serum (21 days after inoculation with saline or 10^4 conidia of *A. fumigatus*) on cumulative mortality of 8-week-old BALB/c mice challenged i.v. with 10^6 viable conidia.

appropriate gating, and spontaneous oxidation of 2',7'-dichlorofluorescin to fluorescent 2',7'-dichlorofluorescein was excluded by subtracting the fluorescence of macrophages loaded with 2',7'-dichlorofluorescin diacetate but not treated with PMA. Data were acquired for 10,000 cells per sample.

Killing of hyphae by PMNs. The effect of preinfection on killing of hyphae by PMNs was assessed in vitro. Heparinized blood was obtained from mice by cardiac puncture and diluted fourfold with sterile isotonic saline. Eight milliliters of blood was gently layered on 3 ml of Lympholyte-M (Cedarlane) and centrifuged at 400 × *g* for 30 min. The pellet was resuspended in 1 ml of plasma and 0.4 ml of 4.5% Dextran T 500 (Pharmacia LKB Biotechnology, Uppsala, Sweden), and the suspension was kept for 40 min at 4°C. The top layer was treated twice with 2 ml of distilled water for 1 min and centrifuged at 600 × *g* for 10 min to lyse erythrocytes. The pellet was resuspended in 0.5 ml of isotonic saline and carefully layered on 3 ml of Percoll (Pharmacia). After centrifugation at 200 × *g* for 10 min, the supernatant was discarded, and the cells were twice washed in isotonic saline and resuspended in supplemented RPMI 1640 medium. This procedure yielded 83% ± 6% PMNs, 14% ± 6% lymphocytes, and 3% ± 1% monocytes, estimated by using Wright's stain. In addition, 16% of the cells were identified as lymphocytes by flow cytometry. Viability of the cells was >98% as determined by trypan blue exclusion. Cocultures of 1.0×10^6 PMNs and 1.0×10^5 hyphae (with an average length of 40 μm) per well of a flat-bottom 96-well tissue culture plate (Nunc) were incubated for 4 h at 37°C with intermittent

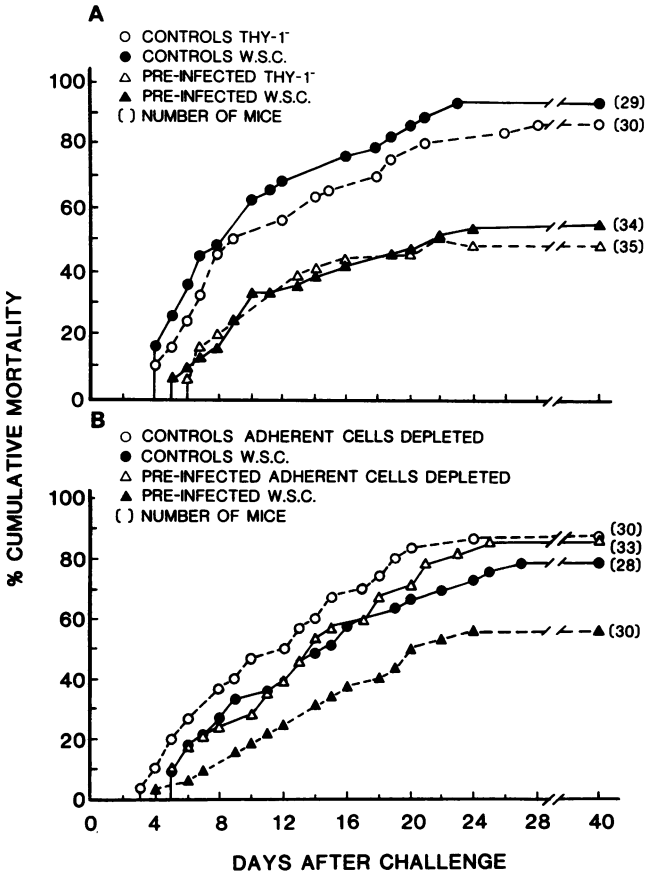

FIG. 4. Effect of depletion of T cells (A) or adherent cells (B) from transferred splenocytes (21 days after inoculation with saline or 10^4 conidia of *A. fumigatus*) on cumulative mortality of naive 8-week-old BALB/c mice challenged i.v. with 10^6 viable conidia. Abbreviations: THY-1⁻, splenocytes depleted of T cells; W.S.C., whole spleen cells.

shaking. The supernatants were discarded, and the hyphae were stained by the MTT procedure.

Statistical analysis. The data were collected over a 2-year period, meticulously checked for internal consistency, and fed to the BMDP Statistical Package (BMDP Statistical Software, Inc., Los Angeles, Calif.). The Kaplan and Meier (22) product limit estimate was used to analyze survival data and plot the survival function. The Breslow (generalized Wilcoxon) and Mantel-Cox (generalized Savage) methods were applied to compare the survival functions of experimental and control populations. The Mann-Whitney U method of the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, Ill.) was used to analyze the chitin content data of the liver, kidney, and lung.

Means of hydrogen peroxide production by peritoneal and splenic macrophages were compared by using a two-way analysis of variance and the two-sample, two-tailed Student's *t* test for independent samples, respectively.

Differences in phagocytosis were determined by using SPSS and BMDP software packages. Repeated-measure analysis of variance was conducted with two factors, one between (group) and one within (time). Significant interactions ($P < 0.00005$) were further analyzed, and significant differences ($P < 0.01$) between the three group means at fixed times were determined by using the Scheffé multiple-comparison test.

Means of killing of *A. fumigatus* were compared by using the two-sample, two-tailed Student's *t* test for independent samples or the Wilcoxon test.

TABLE 1. Estimation of spleen cell populations before and after depletions

Cell populations	Spleen cell composition (%)			
	Total	T-cell-depleted splenocytes	Plastic-adherent splenocytes	Adherent-cell-depleted splenocytes
T cells	34.4	0	0	37.4
B cells	39.0	75.3	10.2	52.6
Macrophages				
Nonspecific esterase	5.3	9.8	ND[a]	0.1
Mac-1 monoclonal antibody	ND	ND	72.0	ND

[a] ND, not determined.

RESULTS

Animal inoculations and adoptive transfer. In preliminary experiments, three groups of eight 5-week-old male BALB/c mice were inoculated i.v. with 1.0×10^4, 1.0×10^6, or 1.0×10^7 viable conidia of *A. fumigatus* 2085. All mice that received 1.0×10^4 conidia were observed for 1 year and survived. Mice receiving 1.0×10^6 conidia died 3 to 8 days after inoculation, while those receiving 1.0×10^7 conidia all died within 2 days after inoculation. Mice were then preinfected i.v. with 1.0×10^4 viable conidia or saline and challenged i.v. with 1.0×10^6 viable conidia after 7, 15, or 21 days. No protection against challenge was found after 7 days. However, highly significant ($P = 0.0002$) and reproducible protection against challenge was observed after 15 and 21 days (Fig. 1). Mortality was reduced from 90% in control mice to 53% in preinfected mice 40 days after challenge. An assay for the cell wall component chitin, a β-(1,4) polymer of *N*-acetyl-D-glucosamine, was used as a means of quantitating *A. fumigatus* in infected organs. Filamentous fungi cannot be reliably quantitated by serially diluting and plating out organ homogenates. Increased survival of mice preinfected 21 days before challenge with 1.0×10^4 viable conidia was correlated (Fig. 2) with decreased content of chitin in the lung, liver, and kidney 4 and 7 days after challenge ($P < 0.05$). In all experiments, invasive aspergillosis was confirmed by culture and histopathology of the kidney, lung, and liver by using the Gomori methenamine silver procedure.

Mice were again preinfected with 1.0×10^4 conidia or saline, and after 21 days, 1.0×10^8 or 2.0×10^8 splenocytes were injected i.v. into naive syngeneic recipients; 2.0×10^8 immune splenocytes conferred significant protection ($P = 0.0001$) against a challenge of 1.0×10^6 conidia i.v. 24 h later (Fig. 3), and mortality was reduced from 83 to 48% 40 days after challenge. Immune serum obtained from the same animals (0.5 ml) offered no protection against challenge ($P = 0.32$ and 0.49 [Wilcoxon and Savage]; Fig. 3), despite the presence of antibody against a hyphal homogenate of *A. fumigatus*, which was absent in the sera of control mice.

The splenic cell population responsible for protection against *A. fumigatus* was determined by removing T cells or adherent cells from control or immune splenocytes and then transferring the remaining cells to naive syngeneic recipients

TABLE 2. Phagocytosis of conidia by peritoneal macrophages of mice infected i.v.

Pretreatment of mice with:	% Of adherent cells containing conidia after[a]:			
	1 h	2 h	3 h	4 h
Saline	58.5 ± 3.1[b,c]	59.9 ± 2.9[b,c,d]	65.4 ± 5.5[b,c,d]	84.0 ± 2.0[b,c]
Conidia (1.0×10^4)	62.0 ± 3.9[c]	72.0 ± 2.2[c,d]	78.5 ± 2.1[c,d]	86.3 ± 2.4[c]
Saline	55.0 ± 5.7[b,c]	58.7 ± 3.9[b,c,d]	65.0 ± 6.6[b,c,d]	82.5 ± 7.5[b,c]
Conidia (5.0×10^5)	52.1 ± 6.0[c]	72.5 ± 5.6[c,d]	77.0 ± 4.9[c,d]	81.2 ± 11.9[c]

[a] Mean ± standard deviation of 10 experiments.
[b] No significant difference ($P = 0.29$) between two saline groups by repeated-measure analysis of variance.
[c] $P < 0.00005$ between saline and preinfected groups by repeated-measure analysis of variance.
[d] $P < 0.01$ between saline and preinfected groups by Scheffé multiple-comparison test.

challenged i.v. with *A. fumigatus*. Protection was maintained after depletion of T lymphocytes (Fig. 4), and depletion of T cells from splenocytes of control and preinfected mice had no significant effect on mortality (93 and 87%, and 53 and 49%; $P = 0.30$ and 0.29, and 0.81 and 0.76 [Wilcoxon and Savage], respectively). However, protection was abolished after removal of adherent cells (Fig. 4). Depletion of adherent cells from splenocytes of preinfected mice produced a significant increase in mortality (60 and 85%; $P = 0.025$ and 0.01 [Wilcoxon and Savage], respectively), which was not observed when adherent cells were removed from splenocytes of control mice (79 and 87%; $P = 0.20$ and 0.22, respectively).

Estimation of splenic cell populations before and after depletions showed complete removal of T cells and enrichment in B cells and macrophages after treatment with associated Thy-1 antiserum and complement (Table 1). Thirteen percent of spleen cells were composed of adherent cells, and a majority (72%) of them were identified as macrophages by using Mac-1 monoclonal antibody.

Production of hydrogen peroxide, phagocytosis of conidia, and killing of conidia or hyphae by peritoneal macrophages. Despite incubation of splenocytes from control or preinfected mice on 3 successive days to minimize empty spaces in plastic petri dishes, the numbers of adherent cells were insufficient for measurement of H_2O_2 production in vitro by the method of Pick and Keisari (34). Peritoneal macrophages were thus initially used to estimate this correlate of the activated state (30). Production of hydrogen peroxide by peritoneal macrophages of control mice was not statistically different from that of mice preinfected with 1.0×10^4 or 5.0×10^5 conidia of *A. fumigatus* i.v. 21 days earlier, with or without stimulation with PMA.

Phagocytosis of conidia by peritoneal macrophages from mice pretreated with 1.0×10^4 or 5.0×10^5 conidia of *A. fumigatus* i.v. was significantly enhanced after 2 and 3 h of incubation compared with that of saline controls (Table 2) but was not significantly different after 4 h. In addition, the percentages of phagocytic adherent cells containing one or six or more conidia after 2 and 3 h of coculture were, respectively, decreased and increased in pretreated animals compared with that of saline controls ($P < 0.01$, Scheffé multiple-comparison test). Phagocytosis of conidia by peritoneal macrophages was also significantly increased in mice pretreated with 5.0×10^7 conidia intratracheally 21 days earlier (Table 3). However, peritoneal macrophages from control mice or animals preinfected i.v. or intratracheally were unable to kill conidia or hyphae in vitro. Indeed, 89.6 and 90.0% of adherent peritoneal cells from control mice or animals infected intratracheally contained conidia after 4 h of coculture, and in both groups of animals, the numbers of viable conidia after a comparable period of coculture (4.5 h) were not significantly different from those in control wells containing conidia alone ($P = 0.70$ and 0.72; 6 to 11 experiments per group).

Production of hydrogen peroxide, phagocytosis of conidia, and killing of conidia or hyphae by splenic macrophages. Phagocytosis of conidia by splenic macrophages was significantly increased in mice preinfected by the i.v. route, compared with that of saline controls, after 1 to 3 h of coculture (Table 4; Fig. 5). In addition, significant killing of conidia by splenic macrophages was demonstrated after 5 h of coculture, but the killing was not significantly different between mice preinfected i.v. and uninfected controls (Table 5 and Fig. 6). Digestion of conidia by splenic macrophages was confirmed by electron microscopy (Fig. 7). Splenic macrophages, however, failed to attach to or to kill hyphae in vitro. Production of H_2O_2 by splenic macrophages, estimated as the percentage of fluorescent cells after pretreatment with 2′,7′-dichlorofluorescin diacetate, was significantly decreased ($P = 0.005$) in mice preinfected with 1.0×10^4 conidia i.v. 21 days earlier (6.6% ± 4.1%) compared with that of saline controls (13.7% ± 6.8%).

Killing of hyphae by PMNs. The percentage of killing of hyphae by PMNs after 4 h of coculture in vitro in mice preinfected with 1.0×10^4 conidia i.v. 21 days earlier (27% ± 9%) was not significantly different ($P = 0.17$) from that of uninfected controls (22% ± 6%). Greater than 97% of

TABLE 3. Phagocytosis of conidia by peritoneal macrophages of mice infected intratracheally

Pretreatment of mice with:	% Of adherent cells containing conidia after[a]:			
	1 h	2 h	3 h	4 h
Saline	43.4 ± 4.9	76.8 ± 5.0	81.4 ± 3.8	89.6 ± 2.9
Conidia (5.0×10^7)	67.6 ± 5.5[b,c]	83.8 ± 4.6[b,c]	89.0 ± 2.6[b,c]	90.0 ± 3.4[b]

[a] Mean ± standard deviation of five experiments.
[b] $P < 0.00005$ between saline and preinfected groups by repeated-measure analysis of variance.
[c] $P < 0.05$ between saline and preinfected groups by Student's t test.

TABLE 4. Phagocytosis of conidia by splenic macrophages

Pretreatment of mice with:	% Of adherent cells containing conidia after[a]:		
	1 h	2 h	3 h
Saline	10.8 ± 4.8	14.5 ± 8.8	10.7 ± 5.7
Conidia i.v. (1.0×10^4)	17.0 ± 8.1[b]	23.6 ± 14.8[b]	19.9 ± 14.5[b]

[a] Mean ± standard deviation of 12 to 13 experiments.
[b] $P = 0.03$ between saline and preinfected groups by repeated-measure analysis of variance.

TABLE 5. Killing of conidia by splenic macrophages

Pretreatment of mice with:	% Killing of conidia after[a]:		P^b
	1 h	5 h	
Saline	4.7 ± 3.2	31.1 ± 18.2	0.03
Conidia i.v. (1.0×10^4)	5.4 ± 4.5[c]	30.9 ± 7.9[c]	0.03

[a] Mean ± standard deviation of six experiments.
[b] Determined by Wilcoxon test.
[c] No significant difference between saline and preinfected groups at 1 h ($P = 0.75$) and 5 h ($P = 0.97$).

untreated control hyphae were viable, whereas hyphae treated with 10% formaldehyde were uniformly killed.

DISCUSSION

A number of studies have substantiated the pivotal role of innate defense mechanisms in protection against invasive aspergillosis (14–17, 25, 27, 37, 38–40, 45). Pulmonary alveolar macrophages rapidly eradicate inhaled conidia in the normal host, while PMNs and monocytes kill hyphae primarily through the myeloperoxidase-H_2O_2-halide system and also by myeloperoxidase-independent oxidative or nonoxidative mechanisms.

Experiments demonstrating increased resistance to lethal i.v. infection with *A. fumigatus* conidia in cortisone-treated (24) or untreated (42) mice preinfected with a sublethal dose of conidia and protection of turkeys inoculated subcutaneously with a killed *A. fumigatus* germling vaccine against subsequent aerosol challenge (36) led us to speculate that acquired immunity may also contribute to host defense against *Aspergillus* infection later in the course of disease. The immune response elicited by the fungus may evolve substantially from the initiation of infection through progressive fungal growth in deep organs.

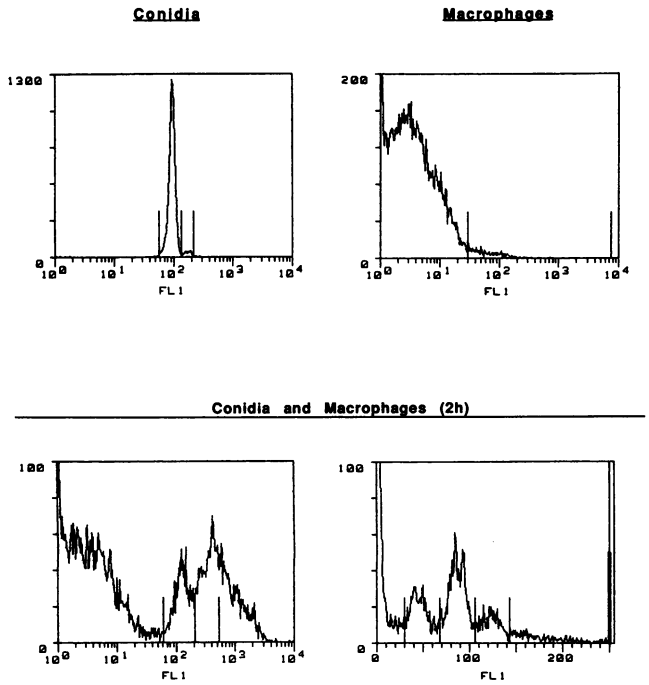

FIG. 5. Phagocytosis of *A. fumigatus* conidia by murine splenic macrophages. Fluorescein-labeled conidia were readily distinguished from background autofluorescence of macrophages. After 2 h of coculture, histograms identified macrophages containing one, two, or three conidia.

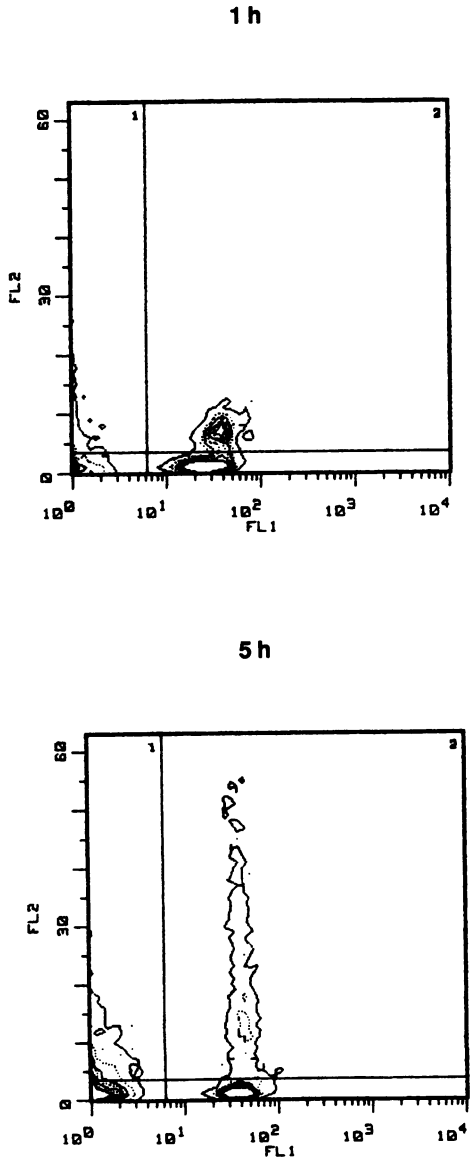

FIG. 6. Killing of *A. fumigatus* conidia by murine splenic macrophages. Green (fluorescein isothiocyanate) and red (propidium iodide) fluorescence are represented by FL1 and FL2, respectively. Low uptake of propidium iodide by conidia after 1 h of coculture was greatly enhanced at 5 h.

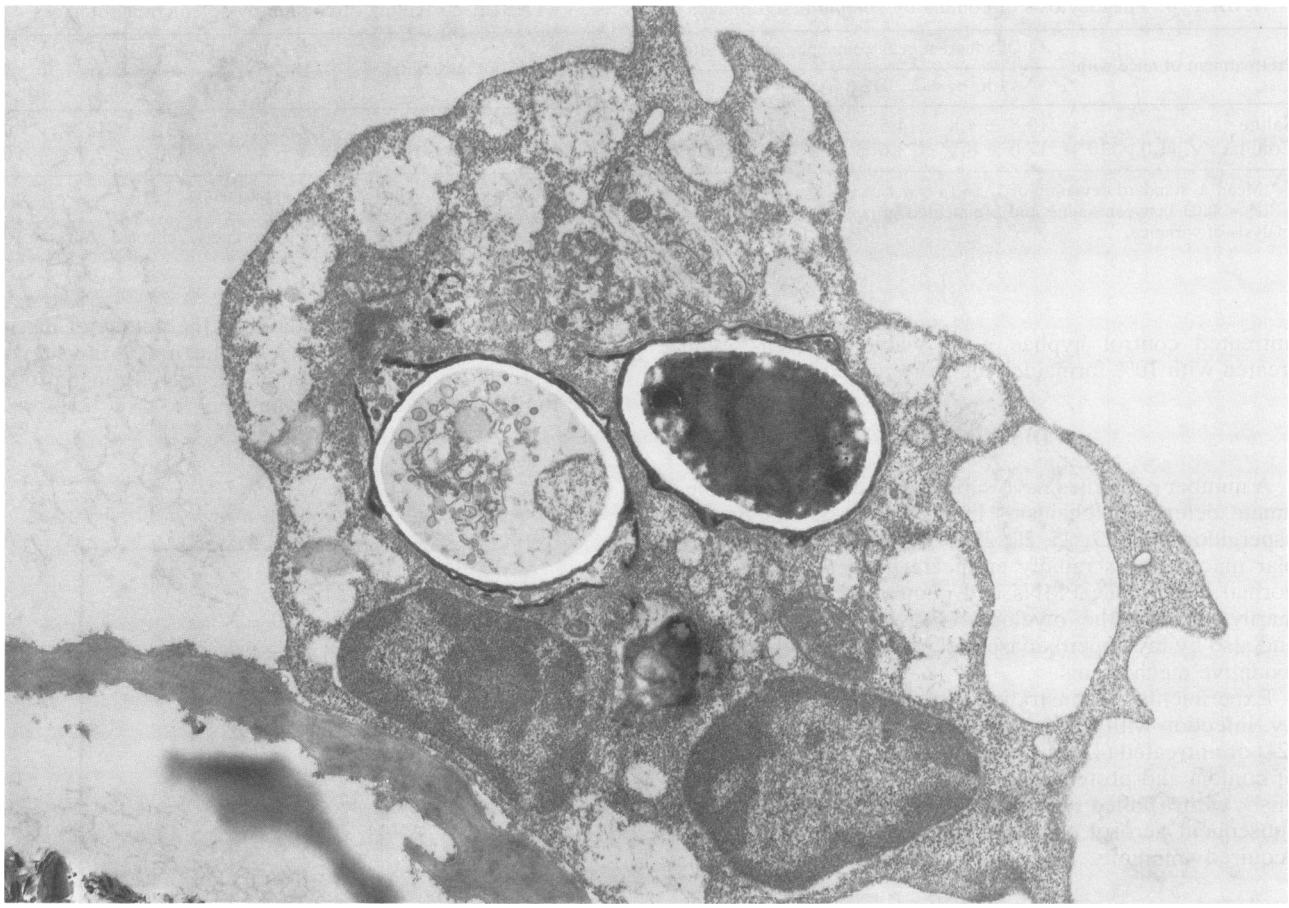

FIG. 7. Digestion of conidia by splenic macrophages after 3 h of coculture. Magnification, ×17,100.

Results reported in this study confirm earlier observations of the protective effect of sublethal i.v. infection on later lethal i.v. challenge with *A. fumigatus* conidia (24, 42, 46) and demonstrate that removal of activated splenic macrophages from total splenocytes abolishes protection of naive syngeneic recipients against i.v. challenge. Although augmented H_2O_2 production represents a biochemical correlate of the activated state (30), lymphokine-mediated macrophage activation against intracellular (10, 31, 41) or extracellular (41) targets may occur without triggering respiratory burst mechanisms. This interpretation was supported by increased phagocytosis of *A. fumigatus* conidia by peritoneal or splenic macrophages from preinfected mice and is consistent with in vitro studies which suggested that killing of conidia by macrophages is mediated by nonoxidative mechanisms (40), possibly the defensin cationic peptides (20, 27).

Activation of macrophages, first described in vivo and then in vitro with lymphokines, has been demonstrated for fungicidal activities against *Candida albicans* (5, 6, 8, 12), *Cryptococcus neoformans* (33), *Histoplasma capsulatum* (47–50), *Blastomyces dermatitidis* (8, 9), *Coccidioides immitis* (2–4), and *Paracoccidioides brasiliensis* (7). Enhanced clearance of the i.v. challenge dose of conidia by activated macrophages in the liver, spleen, and lung could potentially account for the observed protection of the preinfected mice. Schaffner et al. (39) demonstrated that >96% of conidia injected i.v. can be recovered from these macrophage-rich organs, mainly from the liver. This interpretation is supported by the enhanced phagocytosis of conidia by peritoneal or splenic macrophages from mice preinfected by the i.v. or intratracheal routes, although killing of conidia in vitro was demonstrated only with splenic macrophages. The inability of murine peritoneal macrophages to kill *A. fumigatus* conidia is in agreement with the results of Schaffner et al. (40) and emphasizes the divergent antimicrobial functions of macrophages from different anatomical sites. Because killing of conidia is maintained in anaerobic conditions (40), it was suggested that macrophages kill conidia by nonoxidative mechanisms. However, activation of nonoxidative mechanisms may differ in peritoneal and splenic macrophages. Levitz et al. (27) inferred a role for rabbit defensins (20) MCP-1 and MCP-2 in nonoxidative killing of conidia by alveolar macrophages. Moreover, rabbit peritoneal macrophages lack these proteins (20). Although murine defensin proteins have not been studied directly, mRNA for a typical defensin was found in Paneth cells of the mouse small intestine (32). Whether defensins are present in granules of murine or human macrophages is currently unknown.

It could also be hypothesized that the observed protection results from augmented killing of nonphagocytizable *A. fumigatus* hyphae by activated macrophages after germination of the i.v. challenge dose of conidia. Nonphagocytizable *B. dermatitidis* yeast cells, for instance, are killed by peritoneal macrophages activated with gamma interferon (8), by mechanisms independent of products of the oxidative burst

(9). However, the lack of killing of hyphae by peritoneal or splenic macrophages from control mice or animals preinfected by the i.v. or intratracheal routes makes such a mechanism unlikely. Finally, protection of the preinfected mice could be the consequence of the indirect activation of PMN-mediated killing of hyphae by cytokines such as gamma interferon or tumor necrosis factor alpha produced by activated macrophages. The possibility of such a mechanism is suggested by the observed augmentation of in vitro killing of *A. fumigatus* hyphae (35) or *B. dermatitidis* yeast cells (29) by PMNs after administration of gamma interferon in vivo and the enhanced killing of *C. albicans* hyphae by PMNs pretreated with gamma interferon in vitro (18). However, this hypothesis was ruled out by the equivalent killing of hyphae by PMNs from control or preinfected mice.

Lack of protection by T cells or immune serum from preinfected mice is in agreement with the experimental results of Schaffner et al. (39) and Williams et al. (46), respectively, and concurs with the absence of clinical data establishing a connection between invasive aspergillosis and deficient humoral or T-cell-mediated immunity. A role for antigen-specific T lymphocytes as initiators of the reaction and nonspecific macrophages as effectors of the response is nevertheless possible, since T lymphocytes no longer need to be present once the macrophages are activated. To determine whether the effects were induced by *A. fumigatus* conidia specifically will require the demonstration of macrophage activation in vitro by lymphokine-rich supernatants of antigen-stimulated T lymphocytes.

Taken together, the results indicate that acquired immunity mediated by activated macrophages can be demonstrated in experimental murine aspergillosis. Although present biologically, the relevance of this mechanism against the invasive hyphal form of *A. fumigatus* is doubtful. However, additional studies are needed to determine whether activation of pulmonary alveolar macrophages provides protection against *Aspergillus* conidia.

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