

INDUCTION OF AN IgE RESPONSE IN MICE BY *NIPPOSTRONGYLUS BRASILIENSIS*: CHARACTERIZATION OF LYMPHOID CELLS WITH INTRACYTOPLASMIC OR SURFACE IgE¹

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To study the IgE response to helminthic infections at the cellular level in mice, BALB/c mice were inoculated subcutaneously with 400 third-stage infective larvae of *Nippostrongylus brasiliensis* (Nb) and the duration of infection, changes in the mesenteric lymph node (MLN) and spleen cell number and size, cell surface (s) IgD, IgM, IgE, and Thy-1.2, and intracytoplasmic (c) IgE were studied.

Adult worms were cleared from the intestine of infected mice by 10 days after inoculation (DAI). Between 6 and 16 DAI there was a two to four-fold increase in the cell number and the percentage of large cells in both MLN and spleen. The percentage of cIgE⁺ cells increased from undetectable levels in uninfected mice to as high as 0.5 to 1.0% in both the MLN and spleen of infected mice.

Analysis of cell surface molecules with a fluorescence-activated cell sorter (FACS) revealed that Nb infection induced slight increases in the percentages of B cells and slight decreases in the percentages of T cells in MLN and spleen. More remarkably the percentages of sIgE⁺ cells in Nb-infected mice rose from undetectable levels to 35 to 40% of MLN and spleen cells. Analysis of MLN cells with a dual parameter FACS revealed that at the peak of the response almost all sIgD⁺ and sIgM⁺ lymphocytes were sIgE⁺, whereas few if any T cells bore sIgE. Treatment of MLN cells from Nb-infected mice with pH 4.0 acetate buffer for 1 min removed all detectable sIgE from greater than 90% of sIgE⁺ cells but did not remove the sIgE from cIgE⁺ cells or sIgD or sIgM from cells with these surface isotypes. These data indicate that Nb infection is a potent stimulator of the murine IgE immune system and that it 1) induces a small, but considerable percentage of MLN and spleen B cells to acquire intrinsic sIgE and to synthesize large quantities of IgE, and 2) causes the majority of splenic B cells and almost all MLN B cells to acquire cytophilic sIgE.

Because mice, rats, and humans infected with helminths develop striking increases in their serum IgE antibody levels (1, 2), studies of helminthic infections in these species have contributed considerably to the understanding of the regulation of IgE production and the role of IgE in humoral immunity (3–5).

Studies of both mice and rats indicated that infection with *Nippostrongylus brasiliensis* (Nb)² induces an increase in IgE antibody production to nonparasite antigens (6–8), the cellular basis for the increase in IgE synthesis after parasite infection has been studied primarily in the rat. These studies demonstrated a substantial increase in the number of IgE-bearing B lymphocytes in the lymph nodes that drained the site of infection as well as a concomitant increase in the number of IgE-containing plasma cells in these lymph nodes (9, 10). The initial IgE response is polyclonal; the increase in total IgE found in the sera of infected rats precedes detectable IgE antibody to Nb-derived antigens (11). Because of the great availability of well-defined immunologic reagents that are specific for murine determinants and the availability of inbred, genetically defined, and immune-defective murine strains, as well as the current technology for the production of affinity-purified antibody against monoclonal mouse IgE (12), we wished to extend cellular immunologic studies of the IgE response to Nb infection to this species. In this report, we present evidence that Nb infection in the mouse, as in the rat, induces large increases in lymphoid cell numbers as well as in the numbers of B lymphocytes with surface (s) or intracytoplasmic (c) IgE. We demonstrate that all or most of the detectable sIgE⁺ lymphocytes in Nb-infected mice are B cells, and that greater than 90% of the sIgE⁺ cells in these mice bear cytophilic rather than intrinsic sIgE.

MATERIALS AND METHODS

Animals. Eight-week-old female BALB/c, BALB/c By, and 6-wk-old male C57BL/6 mice were purchased from the Jackson Laboratory, Bar Harbor, ME and were used at 8 to 16 wk of age. Two-kilogram female NZW rabbits were obtained from Dutchland Laboratory Animals, Inc., Denver, PA.

Parasites. A strain of Nb that had been adapted to mice was generously provided by Dr. Richard Jacobson, College of Veterinary Medicine, Cornell University. Parasites were maintained at 23°C in a fecal slurry mixed with an equal proportion of charcoal and peat moss. C57BL/6 male mice were infected at monthly intervals with 400 third-stage infective larvae of Nb (L3) subcutaneously (s.c.). Feces were collected daily on moist paper towels at 7 to 11 days after inoculation (DAI) and were used as a source of L3 after 8 days incubation.

Antibodies. Ascites from mice that harbored the SPE-7 IgE anti-DNP-secreting tumor (kindly provided by Dr. Zelig Eshhar, Weismann Institute, Rehovot, Israel and Dr. Philip C. Fox, Laboratory of Microbial Immunity, National Institute of Dental Research, National Institutes of Health) was absorbed with DNP-bovine serum albumin that had been coupled to CNBr-activated Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). This immunoabsorbent was washed with 0.1 M Tris (pH 8.3) and was eluted first with 3.5 M MgCl₂ and then with 0.1 M acetic acid. Very little protein was

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² Abbreviations used in this paper: c, intracytoplasmic; DAI, days after inoculation; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; HNA, Hanks' balanced salt solution with 10% newborn calf serum and 0.2% NaN₃; L3, third-stage infective larvae of *Nippostrongylus brasiliensis*; MLN, mesenteric lymph node; Nb, *Nippostrongylus brasiliensis*; RaMδ, RaMμ, RaMε, and RaF, rabbit antibodies to mouse IgD, IgM, IgE, and ferritin; s, cell surface; s.c., subcutaneously.

eluted with $MgCl_2$, but approximately 0.5 mg protein/10 ml ascites was eluted with acetic acid. The protein eluted was dialyzed against 0.85% NaCl and was concentrated with an Amicon pressure filtration cell, using a PM 10 membrane. Ouchterlony analysis of the IgE preparation showed a single precipitation line when analyzed by gel double diffusion against antisera specific for mouse IgE (Miles-Yeda, Rehovot, Israel) or whole mouse serum. A 50% saturated $(NH_4)_2 SO_4$ cut was prepared from ascites of mice harboring the IgE anti-ovalbumin-secreting hybridoma 14-205, was dialyzed against 0.85% NaCl, and was coupled to CNBr-activated Sepharose. Rabbit anti-mouse IgE (RaMe) antiserum was prepared by immunizing rabbits twice at monthly intervals s.c. with 100 μ g of the monoclonal mouse IgE (SPE-7) in complete Freund's adjuvant (Difco, Detroit, MI). Animals were then boosted monthly with the same immunogen in incomplete Freund's adjuvant. RaMe antiserum was absorbed with normal mouse serum - Sepharose, IgD κ - (TEPC-1033) Sepharose (13) and IgM κ - (TEPC-183) Sepharose, and was then mixed with normal mouse serum (10 ml/100 ml of antiserum) and incubated overnight at 4°C. The absorbed antiserum was centrifuged for 1 hr at 100,000 $\times$ G to remove soluble complexes and then was affinity purified by adsorption to a column of IgE- (14-205) Sepharose before elution with 3.5 M $MgCl_2$ and dialysis of eluted antibody against 0.85 M NaCl.

F(ab')₂ fragments of affinity-purified RaMe were prepared as described above with the following modifications. Before adsorption and affinity purification, the antiserum was precipitated with 50% saturated $(NH_4)_2 SO_4$. The precipitate was dialyzed against 0.1 M sodium acetate (pH 4.5) and digested with pepsin (Calbiochem-Behring, San Diego, CA) (1 mg/50 mg protein) for 18 hr at 37°C. The peptic digest was dialyzed against 0.1 M Tris buffer (pH 8.3), then centrifuged for 1 hr at 100,000 $\times$ G and absorbed and affinity purified as described above. It was absorbed further with protein A-Sepharose (Pharmacia Fine Chemicals) to remove IgG Fc fragments and undigested antibody molecules. The antibodies prepared were isotype-specific by Ouchterlony analysis and enzyme-linked immunosorbent assay.

Techniques for the preparation of affinity-purified rabbit antibodies against mouse IgD (RaM δ), IgM (RaM μ), and ferritin (RaF) have been described (14).

F(ab')₂ fragments of RaM δ , RaM μ , RaMe, and RaF were labeled with fluorescein isothiocyanate (FITC) (Calbiochem-Behring) to molar ratios of from 1.0 to 3.0 as previously described (14). FITC-labeled monoclonal anti-Thy-1.2 mouse antibody was purchased from New England Nuclear, Boston, MA.

Affinity-purified F(ab')₂ fragments of RaMe and RaF were biotinylated by mixing 1 ml of the antibody solution at 1 mg/ml in 0.15 M NaCl/0.1 M NaHCO₃ (pH 8.0) with 20 μ l of a 10 mg/ml solution of (+)-biotin *N*-hydroxysuccinimide ester (Calbiochem-Behring) in dimethyl sulfoxide (DMSO) (Fisher Scientific Company, Pittsburg, PA). The mixture was incubated for 2 hr at room temperature, and then was dialyzed against 0.15 M NaCl. Texas red-avidin conjugates were prepared by mixing 1 ml of a 10 mg/ml solution of avidin (Sigma Chemical Co., St. Louis, MO) that had been dialyzed against 0.15 M NaCl, with 4 ml of 0.15 M NaCl, 0.5 ml of 1 M NaHCO₃ (pH 9.0), and a solution of 1 mg of Texas red (Molecular Probes Inc., Plano, TX) dissolved in 200 μ l of acetonitrile (Sigma) that had been adsorbed with Davidson Molecular Sieves, type 4A, grade 514 (Fisher Scientific). All solutions were cooled to 0°C before mixing and were kept at 0°C for 4 hr after they were combined. Texas red-avidin was separated from free hydrolyzed Texas red by using Sephadex G-25 gel filtration. The conjugates prepared had A₅₉₆/A₂₈₀ ratios of 0.65 to 1.0.

Analysis of cell volume. Single cell suspensions of mesenteric lymph nodes (MLN) and spleen were depleted of erythrocytes with ammonium chloride lysing buffer (M. A. Bioproducts, Walkersville, MD), and cell volume was determined with a Coulter channelizer (Coulter Electronics, Inc., Hialeah, FL) that had been standardized with an erythrocyte preparation (mean cell volume of 79 μ m³) (Coulter Electronics, Inc.).

Immunofluorescence staining for cell surface antigens. Single cell suspensions were prepared from MLN or spleens, were stained directly with FITC-labeled antibodies (14), and then were analyzed for surface fluorescence with a fluorescence-activated cell sorter (FACS II) (Becton Dickinson, Mountain View, CA) (15, 16). The percentages of specifically stained cells and the median fluorescence intensities (median brightness of positive cells) were determined as previously described (14).

Dual parameter fluorescence analysis. The FACS II was modified for dual parameter analysis by the addition of a krypton ion laser and appropriate electronic components. An electronic interface, designed by the Division of Computer Research and Technology, Computer Systems Laboratory of the NIH, was installed to allow for three parameter data processing. This interface permits the operator to "gate" on one parameter while acquiring the second and third parameters. In all instances, the scatter signal was gated while the two additional parameters were acquired as two single, or one dual fluorescence profile. This allows nonviable cells, cellular debris, and clumps of two or more cells to be excluded from the fluorescence analysis (15).

A detailed explanation of the principles of dual parameter FACS analysis has been published (17). It should be noted, however, that cells passing through the FACS during dual parameter analysis are interrogated by the

argon and krypton ion lasers at different points in the fluid stream. Thus, the green (argon laser) and red (krypton laser) generated fluorescence signals (fluorescence 1 and 2) from one cell appear as distinct electronic impulses separated in time by 50 msec. These signals are detected by two different photomultiplier tubes that have filters placed before them to exclude signals from the other channel, and preclude crossover between the two fluorescence signals. The electronic signals generated by this system are stored and retrieved via a PDP-11 computer (Digital Equipment Corp., Manyard, MA) with software developed by the Division of Computer Research and Technology, Computer Systems Laboratory of the NIH.

Fluorescence staining for *cIgE*. One-half milliliter aliquots of an MLN or spleen cell suspension (5×10^5 cells/ml) in Hanks' balanced solution with 10% newborn calf serum and 0.2% NaN₃ (HNA) were centrifuged onto glass slides with a Shandon Southern cytospin cytocentrifuge (Shandon Southern Instruments, Sewickly, PA) for 5 min at 65 $\times$ G. Slides were air-dried, fixed for 30 min in absolute methanol at 4°C, dried, and then stained for 30 min at room temperature in a humid chamber with approximately 50 μ l of FITC-labeled affinity-purified F(ab')₂ fragments of RaMe (100 μ g/ml in HNA). Stained slides were washed three times for 5 min each with phosphate-buffered saline and one time with deionized water. Slides were then air-dried, mounted under glycerol, and examined by fluorescence microscopy using a Leitz Ortholux phase contrast/fluorescence microscope with 6.3 $\times$ eyepieces and a 63 $\times$ 1.4 NA oil immersion objective for enumeration of cells with bright intracytoplasmic fluorescence. Five hundred to 1000 cells on each slide were examined. In preliminary experiments, no cells with bright intracytoplasmic fluorescence were seen when cytocentrifuge preparations of MLN and spleen cells were stained with FITC-labeled F(ab')₂ fragments of RaF to determine background fluorescence.

Acid treatment. MLN cells were treated with 0.05 M acetate buffer (pH 4.0) containing 0.085 M NaCl, 0.005 M KCl, and 1% newborn calf serum for 1 min on ice as described by Kumagai *et al.* (18). Tris buffer (0.1 M) (pH 7.4) with 1% newborn calf serum was added to the suspension to neutralize the acid to the range of pH 7.3-7.4. Cells were then centrifuged at 400 $\times$ G for 10 min and washed two more times in HNA. Microscopic analysis of cells mixed with a solution of trypan blue after acid treatment showed no decrease in viability.

RESULTS

Kinetics of the MLN and spleen cell number and cell size changes during *Nb* infection. Single cell suspensions were prepared from the MLN and spleens of groups of three BALB/c mice that had been infected with 400 L3 s.c. 6, 8, 10, 12, 14, or 16 days before sacrifice. Cell number and size were determined by using a Coulter Counter and Channelizer. By 6 DAI, the number of MLN cells increased to 82×10^6 /mouse as opposed to 19×10^6 /mouse in the uninfected control group. This fourfold increase was persistent through 16 DAI (Table I). The number of spleen cells also increased considerably and peaked 14 DAI at 209×10^6 cells/mouse compared to 95×10^6 cells/mouse in the uninfected animals. Adult worms were cleared from the infected mice by 10 DAI. Increases in the percentages of large cells ($> 429 \mu$ m³) were also

TABLE I
Effect of *Nb* infection of MLN and spleen cell number and size*

Day of Infection	Organ Studied	Cells/Mouse $\times 10^6$	Percent of Cells with Volume (μ m ³)		
			113-248	253-423	429-626
0	MLN	19.4	87.9	10.5	1.6
6		82.3	82.3	15.2	2.5
8		73.6	73.6	22.0	4.4
10		77.2	77.2	18.3	4.5
12		75.4	75.4	19.8	4.8
14		75.4	75.4	19.8	4.8
16		79.5	79.5	16.8	3.7
0	Spleen	95.0	81.1	17.0	1.9
6		165.6	78.0	18.8	3.2
8		164.0	69.9	25.3	4.8
10		175.6	71.0	24.5	4.5
12		177.6	69.0	26.5	4.5
14		208.8	70.5	25.0	4.5
16		168.8	73.7	22.4	3.9

* Three mice/group were inoculated at different times with 400 L3 s.c. and the groups were sacrificed at 6, 8, 10, 12, 14, 16, DAI. A control group (day 0) was sacrificed without being infected. Single cell suspensions were prepared from MLN and spleens, pooled, and analyzed for cell number and size with a Coulter Counter and Coulter Channelizer.

noted in both MLN and spleen by 6 DAI; these increases reached a plateau at approximately three times the baseline values for uninfected mice.

Effect of varying dose of inoculating larvae on MLN and spleen cell size and number. BALB/c mice (three/group) were each inoculated with 0, 100, 200, 400, or 600 L3 s.c. and were sacrificed 12 DAI. There was a considerable increase in MLN and spleen cell number in mice that received 100 or more L3. The greatest MLN response was observed in mice that received 600 L3; these mice had a fivefold increase in MLN cell number and a three to fourfold increase in the percentage of large MLN cells. Smaller increases in spleen cell number were observed in all infected mice; the largest increase in the percentage of large spleen cells was seen in mice infected with 600 L3 (data not shown).

Surface marker analysis of MLN and spleen cells after inoculation with Nb. MLN and spleen cells from control and Nb-infected mice were stained with FITC conjugates of F(ab')₂ fragments of affinity-purified RaM δ , RaM μ , RaM ϵ , RaF (control

for nonspecific staining) antibodies or with an FITC-labeled monoclonal anti-Thy-1.2 antibody and were analyzed on the FACS. Fluorescence profiles of normal and infected MLN and spleen cells staining with RaM ϵ are shown in Figure 1. Cells from uninfected mouse MLN or spleen stained identically with RaM ϵ or RaF (Fig. 1b and d); thus, there were few or no sIgE⁺ lymphoid cells in these mice. In contrast, considerable percentages of both MLN and spleen cells from Nb-infected mice stained specifically with RaM ϵ (Fig. 1a and c). Table II summarizes the changes in surface markers on MLN and spleen cells 6 to 16 DAI with 400 L3. During this period, there was a small increase in the percentage of sIgD⁺ cells and a somewhat greater increase in the percent of sIgM⁺ cells in the MLN. This increase in sIgM⁺ and sIgD⁺ cells was reflected in an approximately 5 to 10% decrease in the percentage of T cells in MLN of infected animals. By 6 DAI, 5.8% of MLN cells bore small but detectable quantities of sIgE (Table II). By 12 to 16 DAI the percentage of MLN cells with sIgE matched the percentage of sIgM⁺ cells, and the average quantity of sIgE on

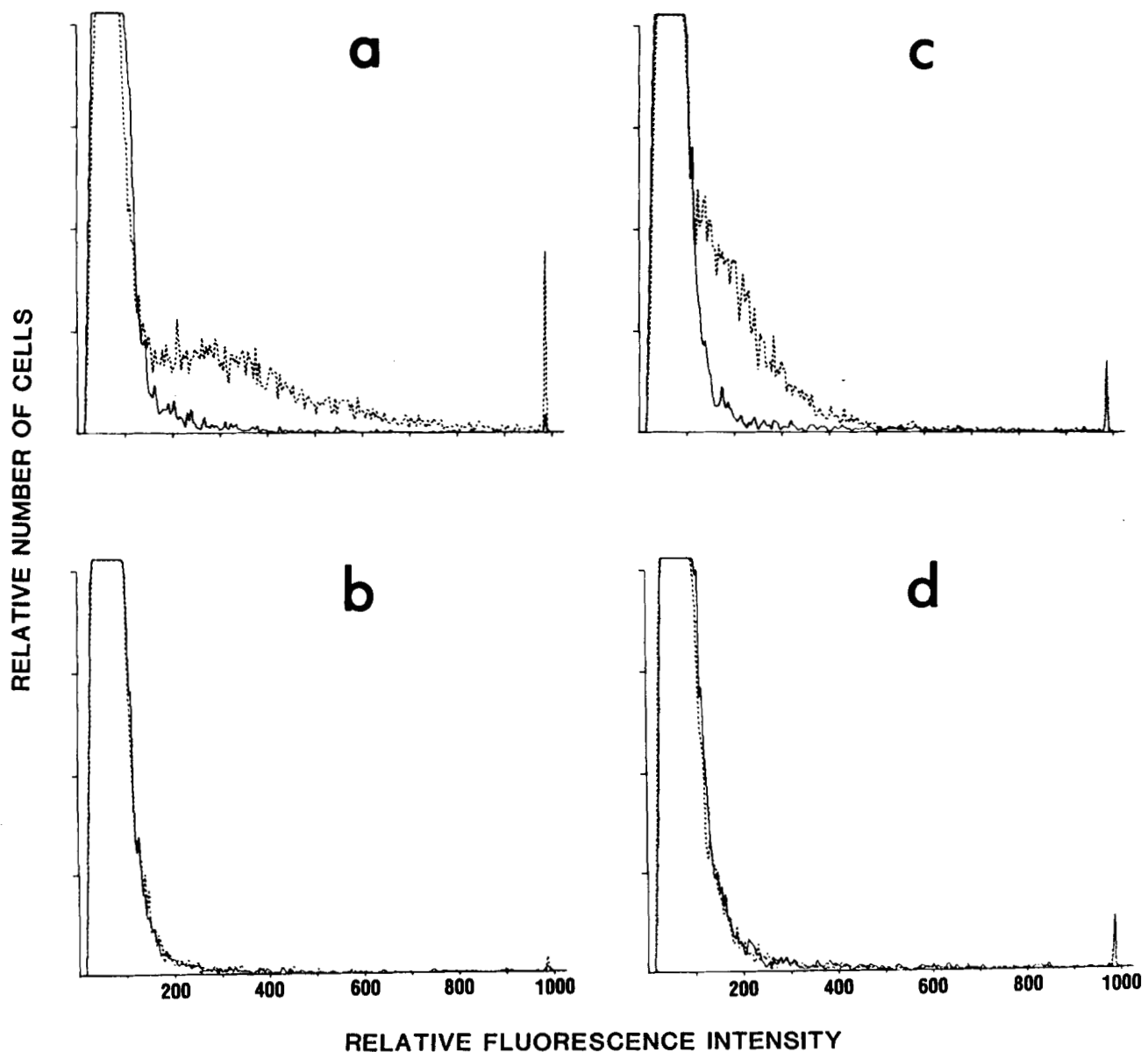


Figure 1. Nb infection induces the appearance of sIgE⁺ lymphocytes. Fluorescence profiles of MLN (a) and spleen (c) cells from mice 12 DAI with 400 L3, and MLN (b) and spleen (d) cells from control animals, stained with FITC-RaM ϵ (dotted lines) or with the control antibody, FITC-RaF (solid lines). In these fluorescence profiles the fluorescence intensity increases along the x axis and the relative number of cells at each channel of fluorescence intensity is shown on the y axis. The cells shown on each panel were analyzed on the same gain.

these cells, judged by the median intensity of surface fluorescence, was three to fourfold greater than that observed on slgE^+ cells 6 DAI. In the spleen, slgE^+ cells were first noted 8 DAI, and increased from 4.8% to 35.5% of the total spleen cell population from day 8 to day 16. Throughout the course of the Nb infection, the average fluorescence intensity of slgE^+ spleen cells was considerably lower than that of slgE^+ MLN cells from the same mice (Table II; compare Fig. 1c with 1a). Varying the dose of L3 (100, 200, 400, or 600/mouse) indicated that inoculation with 400 to 600 L3 induced maximal percentages of slgE^+ MLN and spleen cells (data not shown).

Effect of inoculation with Nb on IgE production. IgE synthesis during Nb infection was determined by enumerating MLN and spleen cells containing large amounts of clgE . In uninfected animals, less than 0.1% of cells stained positively for clgE . clgE^+ cells were first noted 8 DAI with 400 L3 in MLN and 10 DAI in spleen. The percentage of clgE^+ cells peaked at 0.6% at 10 to 12 DAI in both MLN and spleen (Table III).

Analysis of slgE^+ cells. To determine whether B and/or T lymphocytes from the MLN of mice infected with Nb bore slgE , we first stained these cells with FITC-labeled antibodies to one or more cell surface markers. The percentage of those cells that were specifically stained by a mixture of $\text{RaM}\epsilon$, $\text{RaM}\mu$, and $\text{RaM}\delta$ did not differ from the percent stained by $\text{RaM}\epsilon$ (data not shown). This suggested that almost all slgE^+ MLN cells were B lymphocytes. To confirm this finding, MLN cells from infected mice were stained with biotinyl- $\text{RaM}\epsilon$ plus FITC- $\text{RaM}\delta$ or FITC-anti-Thy-1.2, and then washed and counterstained with Texas red-avidin. These cells were then simultaneously analyzed for surface red and green fluorescence with a FACS. As is shown in Figure 2, almost all slgD^+ MLN cells also stained positively for slgE (Fig. 2A), whereas MLN cells that were stained by anti-Thy-1.2 were not detectably stained by $\text{RaM}\epsilon$ (Fig. 2C). Thus, slgE is detectable on most B cells, but on few, if any, T cells obtained from the MLN of infected mice.

Nature of the slgE on B lymphocytes. To determine whether the slgE found on MLN B cells from Nb-infected mice was intrinsic or cytophilic, MLN cells were incubated for 2 or 18 hr in Mishell-Dutton medium at 37°C and were then stained with FITC-labeled $\text{RaM}\delta$, $\text{RaM}\mu$, $\text{RaM}\epsilon$, or RaMF . The great majority of incubated cells lost most of their slgE , but did not lose their slgD or slgM (Fig. 3). Although this suggested the IgE on the surface of most slgE^+ B cells was cytophilic rather than intrinsic, it remained possible these cells experienced metabolic changes in culture that caused them to stop synthesizing IgE. To rule out this possibility, we studied the effect of treating MLN cells with cold acetate buffer for 1 min, because such treatment has been shown to induce the loss of cytophilic IgG by human peripheral blood cells (18). As is shown in Figure 4, treatment for 1 min with acetate buffer (pH 4.0) at 0°C removed detectable slgE from more than 90% of slgE^+ cells but no slgM or slgD from these cells. This finding suggested most slgE was cytophilic rather than intrinsic, but was also compatible with the possibility that acid treatment destroys IgE. The latter possibility was examined by analyzing clgE^+ cells for slgE before and after acid treatment, because plasmotlasts frequently bear the same Ig isotype on their surface as they secrete. MLN cells from Nb-infected mice were stained before and after acid treatment for surface IgE with biotinylated $\text{RaM}\epsilon$ plus Texas red-avidin, then were fixed and stained for clgE with FITC- $\text{RaM}\epsilon$ and examined under the fluorescence microscope. As seen in Table IV, the majority of clgE^+ MLN cells stained positively for slgE ; this staining was not substantially affected by acid treatment. These data strongly suggest the

TABLE II

MLN and spleen cell surface markers during Nb infection ^a					
Day of Infection	Organ Studied	Percent (Median Fluorescence Intensity)			
		slgD	slgM	slgE	Thy-1.2
0	MLN	30.6	29.7	<3.0	61.1
6		35.9	35.1	5.8 (100)	58.4
8		41.0	41.7	20.3 (132)	51.1
10		36.0	38.7	33.7 (252)	56.8
12		36.5	41.1	38.8 (288)	52.4
14		32.5	37.3	37.7 (356)	53.4
16		37.7	40.0	39.6 (264)	49.7
0	Spleen	52.4	55.3	<3.0	27.3
6		48.4	49.6	<3.0	31.7
8		47.1	51.0	4.8 (96)	24.9
10		47.3	50.7	6.9 (104)	23.5
12		51.1	55.1	31.1 (152)	28.1
14		44.7	50.1	28.5 (124)	28.2
16		56.0	59.0	35.3 (132)	27.4

^a Single cell suspensions were prepared from MLN and spleens from mice inoculated with 400 L3. Cells were stained with FITC-labeled antibodies specific for IgD , IgM , IgE , Thy-1.2, or ferritin (control) and were analyzed with a FACS for surface fluorescence and median fluorescence intensity.

TABLE III

Effect of Nb infection on the percent of clgE^+ cells ^a		
Day of infection	Percent of clgE^+ Cells	
	MLN	Spleen
0	<0.1	<0.1
6	<0.1	<0.1
8	0.2	<0.1
10	0.6	0.6
12	0.6	0.4
14	0.4	0.2
16	0.4	0.2

^a Single cell suspensions from MLN and spleens of mice that had been inoculated with 400 L3 were prepared and centrifuged onto glass slides with a cytocentrifuge. Slides were fixed in 100% methanol, stained with FITC- $\text{RaM}\epsilon$, washed, and examined by fluorescence phase microscopy for percentage of cells with bright intracytoplasmic fluorescence.

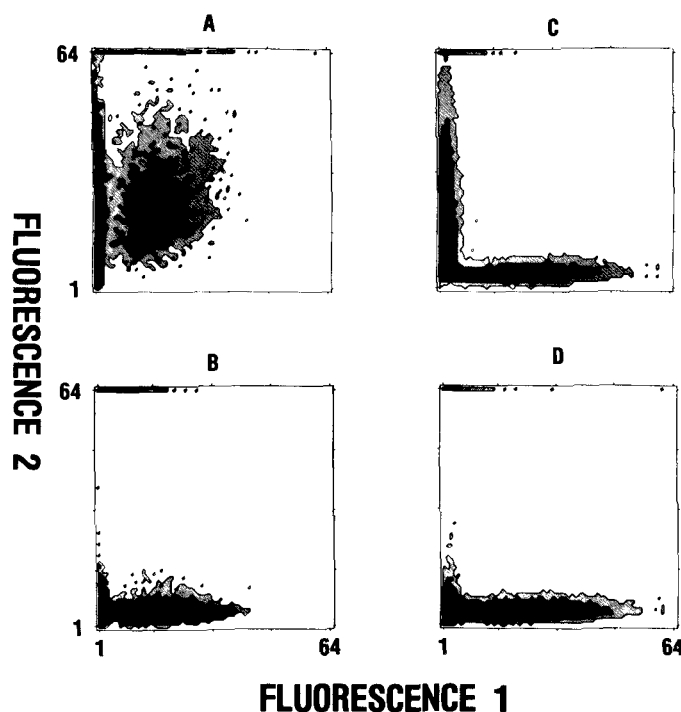


Figure 2. slgE^+ cells are mainly B lymphocytes. MLN cells from Nb-infected mice (400 L3; 12 DAI) or control mice were stained simultaneously with FITC- $\text{RaM}\delta$ or FITC-Thy 1.2 and biotinyl- $\text{RaM}\epsilon$, were counterstained with Texas red-avidin, and then were analyzed with a dual parameter FACS. Fluorescence 1 represents the staining of MLN cells with $\text{RaM}\delta$ (A-infected, B-control) or Thy-1.2 (C-infected, D-control) and fluorescence 2 is the staining with $\text{RaM}\epsilon$ (A, B, C, D). The different shades represent different cell frequencies with the darkest color corresponding to the highest number of cells.

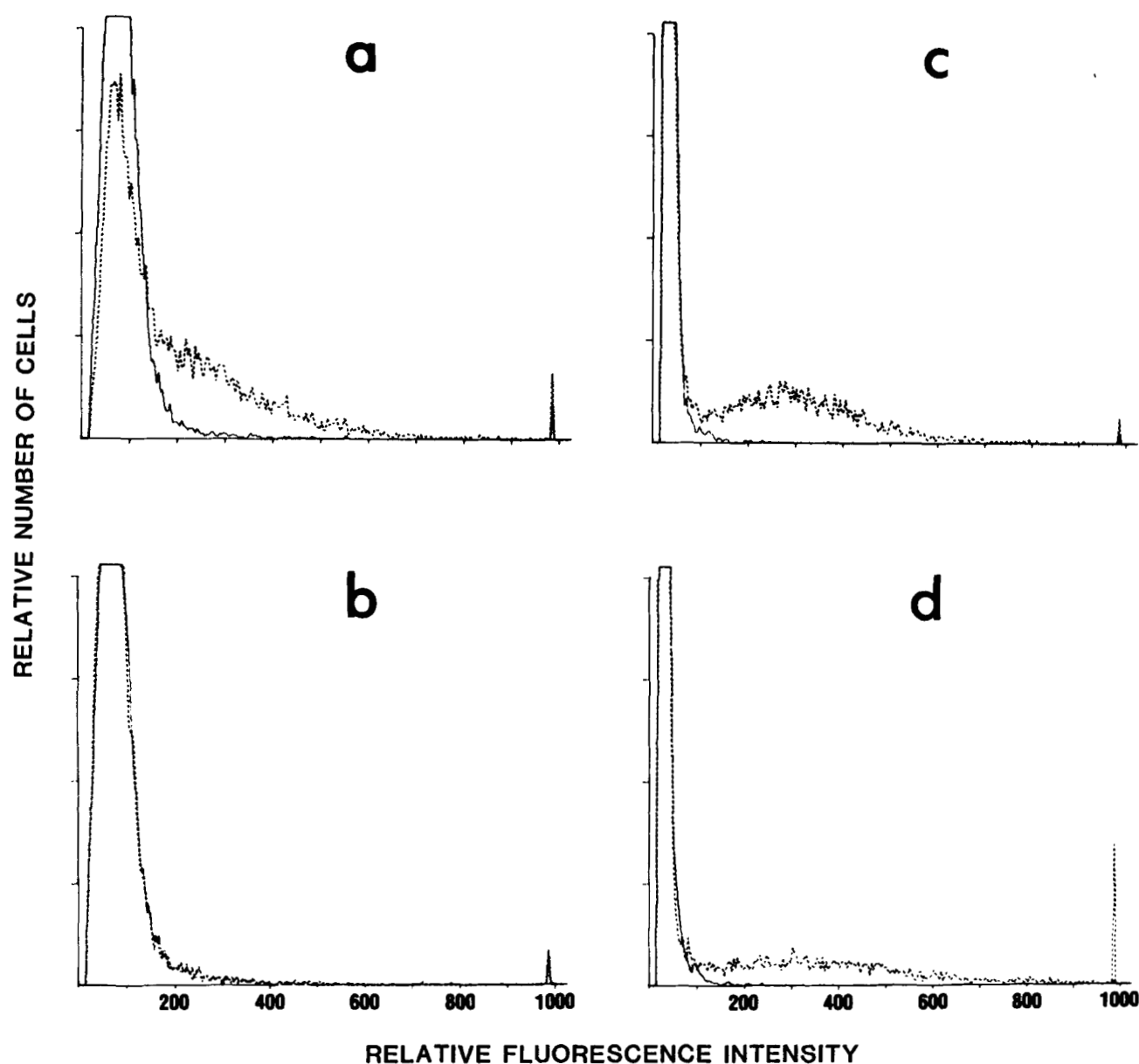


Figure 3. Effect of overnight incubation on MLN cell sIgE and sIgD. MLN cells from Nb-infected mice (400 L3; 12 DAI) were incubated overnight in Mishell-Dutton media at 37°C. Fluorescence cell sorter profiles of MLN cells before incubation stained with FITC-labeled RaMε (a) or RaMδ (c) are indicated with the dotted lines; control antibody RaF staining with the solid line. Other aliquots of MLN cells after overnight incubation were stained with RaMε (b) or RaMδ (d) and were analyzed on the FACS.

majority of cIgE⁺ cells bear intrinsic sIgE, whereas more than 90% of sIgE on sIgE⁺ MLN cells is cytophilic.

To determine whether lymphocytes that retained sIgE after acid treatment also bore sIgM or sIgD, MLN cells from Nb-infected mice were stained after acid treatment with biotinyl-RaMε or RaF and FITC-RaMδ, RaMμ, or RaF, followed by Texas red-avidin, and were then examined by fluorescence microscopy. All cells that stained brightly for sIgE did not have detectable sIgM or sIgD, and only 20% of cells with any detectable sIgE (i.e., approximately 0.5% of the total MLN cells) were stained by FITC-RaMμ or FITC-RaMδ. A similar percentage of sIgE⁺ cells stained simultaneously with FITC-RaF (data not shown). These studies indicate a majority of B lymphocytes with intrinsic sIgE lack sIgM and sIgD, and raise the possibility that no MLN cells exist 12 days after primary Nb infection that simultaneously bear intrinsic Ig of all three isotypes.

DISCUSSION

Our data demonstrate that Nb infection is a potent stimulus of the immune system in the mouse, particularly of the IgE response. Substantial increases in lymphoid cell number and size were seen in the spleen and to even a greater extent, in the MLN of infected mice. Furthermore, by 12 DAI, approximately 60% of splenic B lymphocytes and greater than 95% of MLN B cells bore cell surface IgE and 0.5 to 1.0% of the cells in both of these organs contained large quantities of cIgE, whereas sIgE⁺ and cIgE⁺ cells were undetectable in spleens and MLN of uninfected animals. Similar, although somewhat less dramatic findings have been reported by Ishizaka *et al.* (9) and Urban *et al.* (19) in Nb-infected rats.

A particularly significant and unexpected finding of our study was the observation that brief acid treatment of MLN cells from Nb-infected mice removed sIgE from greater than 90% of the sIgE⁺ cells in these mice, although this treatment failed to

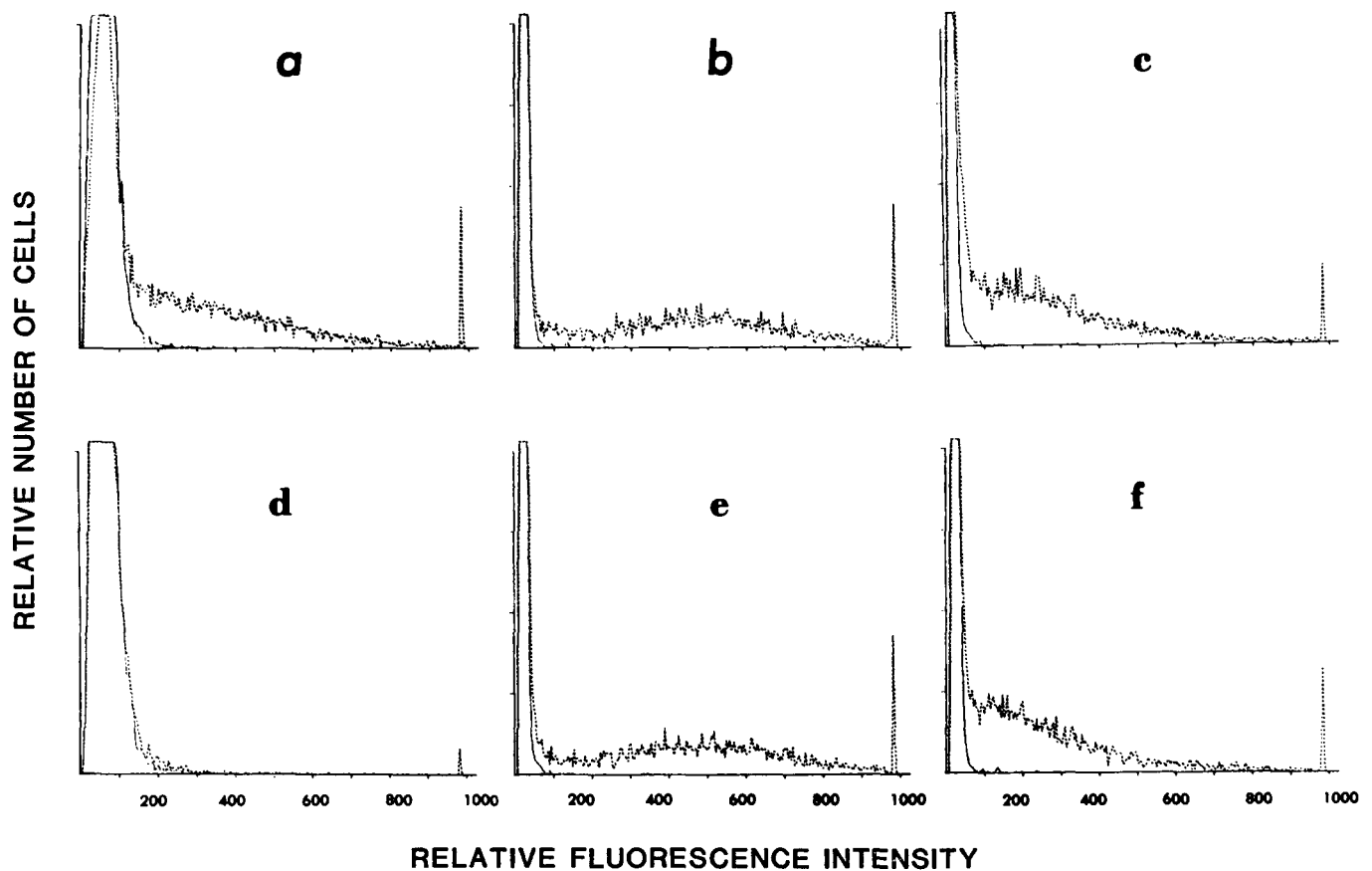


Figure 4. Effect of brief acid treatment on MLN cell sIg. Aliquots of MLN cells from Nb-infected mice (400 L3; 12 DAI) were stained with FITC-labeled RaM ϵ (a), RaM δ (b), or RaM μ (c), and were analyzed on the FACS. After treatment for 1 min with acetate buffer (pH 4.0), other aliquots of these cells were stained with RaM ϵ (d), RaM δ (e), or RaM μ (f), and were analyzed on the FACS. Fluorescence profiles of cells stained with a control antibody (FITC-RaF) are shown as dotted lines; fluorescence profiles of cells stained with FITC-labeled anti-Ig isotype antibodies are shown as solid lines.

TABLE IV
Effect of acid treatment on sIgE⁺ and cIgE⁺ lymphoid cells^a

Nb Infection	Acid Treatment	Percent sIgE ⁺	Percent cIgE ⁺	Percent of cIgE ⁺ Cells that are sIgE ⁺
+	—	37.0	0.56	93.0
+	+	2.7	0.69	76.0
—	—	<0.1	<0.1	—
—	+	<0.1	<0.1	—

^a MLN cells from Nb-infected (400 L3; 12 DAI) and control mice before or after treatment with pH 4.0 acetate buffer for 1 min, were stained with biotinyl-RaM ϵ , counterstained with Texas red-avidin, and centrifuged onto glass slides with a cytocentrifuge. Slides were fixed in 100% methanol, stained with FITC-RaM ϵ , washed and examined by fluorescence microscopy for the red surface and green intracytoplasmic fluorescence.

affect B cell sIgD or sIgM. The observation that this treatment failed to remove sIgE from cIgE⁺ lymphoid cells, which would be expected to bear intrinsic sIgE, strongly suggests that acid-induced loss of sIgE was due to the dissociation of cytophilic IgE from B lymphocytes rather than to the denaturation of intrinsic membrane IgE. This is supported by our finding that greater than 90% of sIgE⁺ MLN cells lose their sIgE after overnight incubation at 37°C. Because almost all B cells from Nb-infected mice bear Fc ϵ R for IgE (Fc ϵ R) (Katona *et al.*, unpublished data) it seems likely that acid-dissociable sIgE represents free or complexed IgE that has bound *in vivo* to an Fc ϵ R.

These results are in agreement with previous reports (20–22) demonstrating the existence of Fc ϵ R on different lymphoid cell types, mainly on B lymphocytes, with the observation of Yodoi *et al.* (23) that Nb infection in rats induced an increase in Fc ϵ R⁺ cells and with the demonstration by Vander-Mallie *et al.* (24)

that the Fc ϵ R cells in Nb-infected mice were mainly B lymphocytes. Furthermore, it supports the observation of Yodoi *et al.* (25) that there is a decrease of Fc ϵ R on lymphoid cells from Nb-infected rats after overnight culture in the absence of exogenous IgE. Recent experiments with FACS analysis of sIgE⁺ cells in Nb-infected rats show, as with Nb-infected mice, that almost all MLN B cells acquire sIgE and that the sIgE on the majority of these cells is cytophilic rather than intrinsic (Urban *et al.*, unpublished data). Earlier experiments (9) showed that approximately 5% of MLN cells from Nb-infected rats reacquired sIgE during overnight culture after pronase treatment. This suggests either Nb infection can induce a greater percentage of cells with intrinsic sIgE in the rat than in the mouse (Table IV) or that the rat B cells can reacquire cytophilic sIgE in culture.

The finding that the IgE on the surface of most MLN B cells of Nb-infected mice is cytophilic rather than intrinsic is important for two reasons. First, IgE binding T cell factors have been described that stimulate or suppress B cell differentiation into IgE-secreting cells (26–28) and the binding of secreted IgE to a B cell Fc ϵ R may have a role in controlling IgE secretion by providing a mechanism for focusing these factors onto B cells. Second, the existence of "triple isotype-bearing" B lymphocytes with intrinsic sIgD, sIgM, and sIgE would appear to be incompatible with theories of molecular mechanisms of isotype switching that predict that B cells would have to delete their genes for the constant region of μ - and/or δ -chains in order to transcribe mRNA for ϵ -chain (29, 30). Our data indicate that a maximum of 0.5% of MLN cells at 12 DAI might bear intrinsic sIgE along with sIgM or sIgD. We cannot rule out the possibility

that these cells synthesize a single mRNA that codes for μ -, δ -, and ϵ -chains. Indeed, cells that synthesize $\gamma 2b$ without having deleted a μ -chain gene have been described (31). It is equally possible, however, that these cells with slgE, slgM, and slgD recently deleted the μ - and/or δ -genes and retain previously synthesized μ/δ mRNA and/or slgM and slgD, at the same time that are actively synthesizing mRNA for ϵ -chain. An additional possibility is that these cells bear cytophilic rather than intrinsic slgE that was incompletely removed by the acid treatment. Any of these possibilities are compatible with reports by Suemura *et al.* (32) and Shigemoto *et al.* (33) that suggest many precursors of IgE-secreting cells bear slgE along with slgM and/or slgD. Finally, because the percentage of post-acid treatment slgE⁺ cells that were stained simultaneously by RaF closely approximated the percentage stained by RaM μ or RaM δ , it is not unlikely that this small percentage of "triple-bearing" cells were damaged by the acid treatment and were stained nonspecifically. In any event, although our data by no means disprove the possibility of ϵ -gene expression without deletion of the μ and/or δ -genes, what was thought to be a major objection to the gene deletion theory is removed.

Another important finding of our study was the failure to detect slgE on T lymphocytes in the MLN or spleens of Nb-infected mice, although the great majority of B cells in these organs were slgE⁺. This was somewhat surprising, because Chen *et al.* (34) obtained evidence that in the murine spleen cultured for 24 hr in the presence of monoclonal IgE, both B and T cells become Fc ϵ R⁺, and Vander-Mallie *et al.* (24) recently reported that approximately 8% of splenic T cells from Nb-infected mice bear an Fc ϵ R. Possibly Fc ϵ R⁺ T cells bear lower quantities of this receptor and/or a less avid form of this receptor than do Fc ϵ R⁺ B cells, so that they would be less able to retain IgE *in vitro* and would be detected as slgE⁺ cells in our assay system. In addition, our dual fluorescence assay system might fail to detect cells that constituted less than 2 to 3% of the total population analyzed. Further investigation of this point is important, because the Fc ϵ R⁺ T cell has been identified as the source of IgE-specific helper factor in the rat (35).

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