Inhibition of Natural Killer Cell Activity by a Soluble Substance Released by Rat Peritoneal Cells

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ABSTRACT

We describe here a soluble substance released by nonadherent cells from the peritoneal cavity of W/Fu rats that markedly inhibits the activity of mouse, rat, and human natural killer (NK) cells. The NK-inhibiting substance (NK-IS) has low molecular weight (<1000), is heat resistant (100°C for 15 min), and is insensitive to nonspecific proteases. NK-IS is produced in the presence of indomethacin (1 to 10 μg/ml), suggesting it is not prostaglandin. The inhibitory effect was seen on unstimulated as well as on cells activated in vivo or in vitro by Corynebacterium parvum. The activity of cells mediating antibody-dependent cell cytotoxicity (K-cells) was also inhibited by NK-IS although to a lesser degree. In sharp contrast, the substance had little effect on lysis mediated by murine or human cytotoxic T-lymphocytes. Production of NK-IS from rat peritoneal cells was significantly greater than by spleen cells. Since the peritoneal cavity is relatively deficient in base-line NK activity compared to spleen, these data suggest that NK-IS may play an in vivo role in the expression of NK cytotoxicity.

INTRODUCTION

NK cells have gained considerable attention recently because of their possible roles in the host defense against malignant neoplasms (12, 15). Tumor cells and a variety of nontransformed cell types, including macrophages, fibroblasts, and thymocytes, are sensitive to NK lysis (13, 18, 21, 28, 30), suggesting additional roles for NK cells in the control of normal cell functions. These attributes of NK cells underscore the need for studying and characterizing the mechanism(s) responsible for their regulation in vivo and in vitro.

These regulatory mechanisms would consist of amplification signals produced when the organism is appropriately stimulated and/or inhibitory signals once the activating stimulus has been eliminated or controlled. Furthermore, since normal lymphohematopoietic tissues are susceptible to NK lysis (13, 18, 21, 30), the mechanisms that inhibit NK activity may be important in preventing the development of uncontrolled autoaggressive disorders. Despite numerous reports on the activity, characteristics, and properties of NK cells, the mechanisms active in their regulation and especially suppression have remained poorly defined and highly controversial (8).

Spontaneously occurring suppressor cell populations have been described among murine thymocytes and human peripheral blood T-cell subsets (19, 20). In addition, certain subpopulations of cells, stimulated by diverse agents, can inhibit NK cytotoxicity. Injection into mice of carrageenan (9), Corynebacterium parvum (24), hydrocortisone (16), and Adriamycin (23) activates suppressor cells that inhibit NK cell activity.

Several of these suppressor cells mediate inhibition of NK cells through secretion of soluble factors. Suppressor cells activated by carrageenan (9) and hydrocortisone (16) produce soluble suppressive substances that inhibit cytotoxicity. Prostaglandins, which are suppressive to NK cytotoxicity (4, 10, 29), may also play a role in suppression by regulatory cells. In addition, certain substances such as estrogens, antiproteases, and carbohydrate molecules effectively inhibit NK lysis (17, 25, 27), although it is unclear what role these chemicals may play in its regulation in vivo.

The study reported herein examines the effects of a soluble mediator released by unstimulated, nonadherent PCs from normal W/Fu rats that has unique properties. This inhibitory substance, designated here as NK-IS, effectively inhibits murine and human NK cells.

MATERIALS AND METHODS

Animals. Eight- to 10-week-old female W/Fu rats were acquired from Microbiological Associates, Inc. (Walkersville, Md.) and rested for at least 10 days prior to their use.

Tumor Lines. The Gross virus-induced lymphoma, G-1 (26) (a generous gift from Dr. R. Herberman, National Cancer Institute), the human K562 tumor line, and the YAC-1 mouse lymphoma were used as NK-sensitive targets. Both lines were passaged in RPMI media supplemented with 10% FCS (Reheis, Phoenix, Ariz.), L-glutamine, and antibiotics. The CS6NTD tumor line (a gift from Dr. Herberman, National Cancer Institute) and Raji B-lymphoblastoid cell line were also passaged in RPMI media supplemented with 10% FCS.

Harvesting of Lymphoid Cells. Animals were sacrificed by cervical dislocation, and PCs were obtained by repeated lavage of the peritoneal cavity with sterile, cold Dulbecco’s PBS (Grand Island Biological Co., Grand Island, N. Y.). Single-spleen cell suspensions were obtained by expression of the organ through a stainless steel mesh. Human PBLs were prepared by separation on Ficoll-Hypaque as described previously (3). All cells were washed 3 times, counted in a hemocytometer, and resuspended to desired concentrations.

In Vivo Augmentation of NK Cytotoxicity. One mg of C. parvum light residue (7) was injected i.p. into W/Fu rats. Five to 10 days following injection, the animals were sacrificed, and PCs and spleen cells were obtained and used as effector cells.

In Vitro Augmentation. Spleen cells and PCs were obtained from normal adult rats and washed 3 times. Twenty-five 10° cells in 10 ml of RPMI media supplemented with 10% FCS, L-glutamine, and antibiotics were cultured with C. parvum light residue in a final concentration of 100 μg/ml. The flasks were kept in a CO₂ incubator at 37°C for 20 hr, after which the cells were harvested, washed 3 times, counted, and used as effector cells.
Complement-dependent Antibody-mediated Cytotoxicity. Raji target cells (10⁴ cells/well) were chromated and incubated with NK-IS and anti-Raji antibody in microtiter plates for 30 min at 4°C. Normal rabbit serum was then added as a source of complement directly to the wells, and the plates were incubated at 37°C for 30 min. The plates were then centrifuged for 10 min, and supernatant was removed and counted in a γ counter. Maximal release was determined by exposing target cells to detergent and spontaneous release by incubation of targets in media alone. Percentage of specific lysis was determined by:

\[
\text{Experimental cpm} - \text{control cpm} \times \frac{100}{\text{Detergent releasable cpm} - \text{background cpm}}
\]

Staphylococcal A-bound Sepharose Column to Remove Immunoglobulin. Staphylococcal protein A, covalently linked to Sepharose CL-4B was obtained from Pharmacia (Uppsala, Sweden), swollen in 10 ml PBS, pH 8.0, containing 0.1% sodium azide and packed into a 1-ml column. The column was washed with buffer, pH 3.0, to free bound material and then equilibrated at pH 8.0. Crude PC supernatant fluid was applied to the column, and the column was then washed with 10 ml of 10 mM PBS, pH 8.0. Fractions of 1 ml were collected and tested for the ability to suppress NK cytotoxicity. As a positive control, we filtered monoclonal IgG antibodies to neuroblastoma and to the human equivalent of Thy 1 antigen through the staphylococcal protein A column. The effluent fractions were tested for specific antibodies using a binding assay with ¹¹¹I-labeled staphylococcal protein A. Background binding was obtained with each of the effluent fractions, suggesting complete removal of specific antibodies (data not shown).

Production of C. parvum Light Residue. The light residue fraction of C. parvum was produced as described previously (7). Briefly, C. parvum strain C6N134, Lot CA 771/B, a generous gift from Richard Tuttle, Ph.D. (Burroughs Wellcome, Research Triangle Park, N. C.), was fractionated by the Westphal method. C. parvum whole cells were added to sterile distilled water and liquified phenol. After stirring for 30 min at 65°C and cooling for 2 hr at 4°C, the extraction mixture was centrifuged at 25,000 × g for 1 hr at 4°C. Pelleted materials (residue) were extracted 2 more times in the original volumes of phenol and water. The insoluble residue was suspended in distilled water and centrifuged at 30 × g for 10 min. The resulting supernatant was designated light residue and was hophylized. The residue consists of 88% of recovered whole C. parvum cells, and the light residue consists of 89% of the residue fraction. By weight, the light residue is 23% protein, 32% fatty acid, and 11% total sugar. We have found consistently that the enhancing properties of C. parvum on NK cytotoxicity due to in vivo or in vitro treatment is enhanced greatly in light-residue fractions.

Treatment of NK-IS with Insoluble Protease. Nonspecific protease from Streptomyces griseus was used bound to carboxymethyl cellulose (Lot 71 F-9550; Sigma Chemical Co.). Two units of bound protease (0.015 g) were washed 3 times with PBS and 1 time with RPMI media and then resuspended in 0.6 ml of either undiluted NK-IS or RPMI media. The mixtures were incubated for 2 hr at 37°C, protease beads were spun down, and supernatant was removed. The supernatant was spun a second time to ensure removal of protease beads and assayed for inhibitory activity to NK cytotoxicity. To ensure that the insoluble protease was active, 0.5 to 1 unit was incubated with BSA for 2 hr at 37°C. After incubation and centrifugation of beads, supernatants were harvested and electrophoresed in sodium dodecyl sulfate on a polyacrylamide gel. The results revealed that the insoluble protease effectively degraded BSA into smaller molecular weight fragments during a 2-hr incubation.

Determination of Molecular Weight. Spectrotr 6 dialysis tubing with molecular weight exclusion size 1000 (Scientific Products) was used. One ml of either NK-IS or RPMI media was placed in dialysis tubing and dialyzed for 16 hr against 5 liters of distilled water. After dialysis, samples were removed from the tubing and reconstituted with 10 × RPMI media to replace dialyzed salts and nutrients. The samples were then tested for inhibitory activity against NK cytotoxicity.
RESULTS

Reduction of NK Cell Activity by NK-IS, a Substance Produced by Rat PCs. Supernatant fluids obtained by overnight culturing of PCs from adult W/Fu rats markedly inhibit murine and human NK cell activity. Eight-week-old rats were sacrificed and their PCs harvested by lavage with cold PBS. The cells were incubated in RPMI media supplemented with l-glutamine and antibiotics for 24 or 48 hr after which cell-free supernatant was obtained and filtered. Table 1 shows that these supernatants contain a soluble factor (or factors), which we have termed NK-IS, that suppresses NK cytotoxicity. Rat spleen cells incubated in NK-IS obtained from PCs of 3 different sets of rats for 1 hr at 37° demonstrated markedly reduced NK activity when tested against the NK-sensitive target G-1. NK-IS obtained after 24-hr incubation of PCs is as suppressive as material obtained after 48 hr of incubation (data not shown). The potency of the NK-IS detected in supernatant fluids was directly related to the concentration of PCs. The most suppressive supernatants were collected from PCs cultured at 10^7/ml for 24 hr. At concentrations lower than 10^6/ml, no NK-IS was detected (data not shown). The effect of NK-IS on rat cells was dose dependent (Chart 1). NK-IS preparations were usually active up to a 1:10 dilution. NK-IS was not directly cytotoxic to murine splenocytes or human PBL. Incubation of NK-IS with these cell populations for up to 24 hr did not result in loss of cell viability, as measured by trypan blue dye exclusion.

Lack of Species Restrictions of Inhibitory Effects of NK-IS. NK-IS obtained from PCs of adult W/Fu rats inhibits mouse and human as well as rat NK effectors. Splenic NK activity of mice directed towards YAC targets and human PBL NK activity towards K562 targets were significantly suppressed by NK-IS (Table 1). These data indicate that the activity of NK-IS is not species restricted and suppresses cytotoxicity directed toward different NK sensitive targets.

The inhibitory effect induced by NK-IS on human and mouse NK effectors was not readily reversible by washing. Unstimulated rat effectors were more dependent on the continuous presence of the inhibitory factor (Chart 1) whereas in vivo or in vitro-activated rat spleen cells or PCs were markedly suppressed even if the NK-IS was washed off after the 1-hr incubation.

NK-IS Suppression of Cytotoxicity Mediated by Activated NK Effectors. NK effectors were activated by either i.p. injection of adult W/Fu rats with 1 mg of C. parvum light residue followed with harvest of spleen cells and PCs 5 days later or by culturing rat spleen cells or PCs overnight with C. parvum light residue (100 µg/ml). Table 2 is a representative experiment. As shown, NK-IS suppressed cytotoxicity mediated by in vitro- and in vivo-activated effectors. Thus, both PC and splenic NK effectors were suppressed after in vivo or in vitro activation by C. parvum.

Effects of NK-IS on Other Cytotoxic Effector Functions. NK-IS preferentially inhibits NK cell activity. The factor had no effect on the cytotoxic activity of spleen cell populations sensitized against (C58NTD) tumor cells in vivo (data not shown). In humans, NK-IS inhibits K-cell as well as NK cell activity while having minimal effects on cytotoxicity mediated by T-lymphocytes (Chart 2). The ability of NK-IS to influence several cytotoxic functions suggested the possibility that this substance acted by reducing target cell release of radiolabeled chromium. To rule this out, we explored the effect of NK-IS on complement-dependent antibody-mediated cytotoxicity. NK-IS did not influence the lysis of 51chromium-labeled cells exposed to specific antibodies and complement while causing marked inhibition of NK activity tested simultaneously (data not shown). These results refute the notion that NK-IS affects cytotoxicity by inhibiting chromium release.

Characterization of NK-IS. Attempts to concentrate NK-IS by Amicon ultrafiltration using membranes that exclude substances with molecular weights of ≥30,000, 10,000, and 1,000 were unsuccessful. In addition, G-25 and G-10 gel filtration indicated the suppressive material resided within the inclusion volumes of both columns, suggesting the inhibitory molecule was quite...
added to chromated K562 for CRA. The preincubating media or NK-IS was either fresh or had been previously incubated at 37°C for 2 hr or incubated with insoluble protease for 2 hr at 37°C. Effectors were washed and added to appropriate targets for NK, antibody-dependent cell-mediated cytotoxic, and T-cell cytolysis assays. O, NK assay; ●, antibody-dependent cell-mediated cytotoxic assay; △, T-cell-mediated lysis; Points, mean for 3 experiments; bars, S.E.

### Table 3
**Evaluation of NK-IS molecular weight**

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>Dialed and reconstituted</th>
<th>% of specific release (effector:target cell ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>–</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>NK-IS</td>
<td>–</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>Media</td>
<td>+</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>NK-IS</td>
<td>+</td>
<td>25 ± 2</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

**NK-IS is not affected by treatment with insoluble proteases**

PBLs were preincubated with media or NK-IS that was fresh or dialyzed through Spectrator tubing with an exclusion cut off of 1,000 followed by reconstitution (see "Materials and Methods"). After incubation, PBLs were added to chromated K562 for CRA.

### Table 4
**NK-IS dilutions**

<table>
<thead>
<tr>
<th>NK-IS dilutions</th>
<th>% of specific release (effector:target cell ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>NK-IS</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>Media</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>NK-IS</td>
<td>25 ± 2</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

**NK-IS is not affected by incubation with indomethacin**

NK-IS was prepared by culturing rat PCs at a concentration of 5 x 10^6/ml in either media alone or media supplemented with indomethacin (1 or 10 µg/ml). For control purposes, media alone or media supplemented with indomethacin (1 or 10 µg/ml) were cultured overnight (with no cells) under identical conditions.

### Table 5
**Production of NK-IS not affected by incubation in the presence of indomethacin**

<table>
<thead>
<tr>
<th>Human effector cells incubated in</th>
<th>Indomethacin added (µg/ml)</th>
<th>% of specific release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50:1</td>
<td>25:1</td>
</tr>
<tr>
<td>Media</td>
<td>33 ± 2</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>Media</td>
<td>1</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>Media</td>
<td>10</td>
<td>40 ± 2</td>
</tr>
<tr>
<td>NK-IS</td>
<td>1</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>NK-IS</td>
<td>10</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>NK-IS</td>
<td>8 ± 1</td>
<td>5 ± 1</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

These results suggest that prostaglandin synthesis during overnight incubation of peritoneal cells plays no or minimal role in the generation of NK-IS.

**Cell Populations Producing NK-IS.** NK-IS is predominantly produced by PCs. There was little effect on cytotoxicity when NK effectors were preincubated in supernatants obtained from rat spleen cells incubated for 24 hr (Chart 3). Plastic nonadherent PCs are the producers of NK-IS. When PCs were depleted of adherent cells by incubation on plastic Petri dishes for 45 min, there was no diminution in the amount of NK-IS produced by nonadherent cells during 24-hr incubation. When the nonadherent cells were adhered a second time under the same conditions, the results were identical, i.e., the nonadherent cell fraction produced NK-IS (Chart 3). By contrast, plastic adherent PCs produced little NK-IS or none at all.

PCs obtained from rats given i.p. injections of PBS 3 days previously were comparable to PCs of unperturbed rats in their ability to produce NK-IS (data not shown). In contrast, PCs from rats given i.p. injections of 1 mg of C. parvum light residue 5 days previously produced less NK-IS during overnight incubation (Table 6). Sera from rats injected i.p. with PBS or C. parvum 5 days previously had minimal effects on NK cytotoxicity (Table 6).

**DISCUSSION**

Resident cells from the peritoneal cavity of rats produce a substance that inhibits NK cell cytotoxicity. This substance acts small. This was confirmed by the experiment presented in Table 3. NK-IS was placed in spectrapor dialysis bags with a molecular weight exclusion size of 1,000 and dialyzed overnight. Suppressive material was lost from inside the bag, indicating that the molecular weight of NK-IS <1,000.

NK-IS is not sensitive to the proteolytic effects of a nonspecific protease. Table 4 demonstrates that incubation of NK-IS with an insoluble protease for 2 hr resulted in no loss of suppressive activity. Polyacrylamide gel electrophoresis indicated that the insoluble protease was effective in degrading BSA into smaller molecular weight fragments during a 2-hr incubation, attesting to the effectiveness of the protease.

NK-IS is effectively produced by peritoneal cells in the presence of indomethacin (1 or 10 µg/ml) (Table 5). These concentrations of indomethacin inhibit prostaglandin synthesis (11).
in a dose-dependent fashion, is species nonrestricted, and inhibits cytotoxicity directed against at least 3 separate NK-susceptible targets.

NK-IS appears to act at the effector cell level. Unlike some high-molecular-weight compounds that nonspecifically inhibit release of radiolabeled chromium from target cells (14), NK-IS had no effect on complement-dependent antibody-induced lysis. The inhibitory action is a temperature-dependent event. Preincubation of effector cells in NK-IS at 4° (instead of 37°) for 60 min resulted in significant reduction in the suppression detected. Supernatants collected following preincubation of effector cells at 4° and 37° exhibited comparable reduction in NK-IS activity, suggesting that the amount of NK-IS bound by effector cells was comparable at the 2 temperatures tested.

Roder et al. (28) have presented data that binding and postbinding events of NK lysis are regulated independently. The mechanism of action of NK-IS is under investigation. Preliminary studies indicate that NK-IS influences postbinding events.  

Local production and activity of NK-IS may account for the variability in NK cytotoxicity detected at different anatomical sites. Spleen cells with high base-line NK cytotoxicity (22) produced significantly less NK-IS than PCs, a population with inherently minimal NK activity supporting an in vivo role for NK-IS in regulation of NK cytotoxicity. If NK-IS is shed primarily (or produced) by NK-sensitive normal cells, it would also explain effects reported by Brunda et al. (5) and those reported in this study. We are uncertain whether the C. parvum activated mouse adherent PCs were less inhibitory of spontaneous or interferon-augmented NK activity than were nonactivated adherent cells. We do not know whether C. parvum activated mouse adherent cells produce less NK-IS than control rats. This correlated with increases in peritoneal NK activity following adjuvant injection. We do not know whether C. parvum directly inhibits NK-IS-producing cells. A likely explanation is that the influx of irrelevant cells into the peritoneal cavity that follows injection reduces the proportion of cells releasing NK-IS. Alternatively, C. parvum may induce the production of mediators that augment NK cytotoxicity and counteract the effects of NK-IS. Brunda et al. (5) reported that C. parvum-activated mouse adherent PCs were less inhibitory of spontaneous or interferon-augmented NK activity than were nonactivated adherent cells. We are uncertain whether the C. parvum effects reported by Brunda et al. (5) and those reported in this manuscript are mediated via the same cellular mechanism. Studies to phenotypically characterize the cells producing NK-IS and the mechanisms whereby C. parvum reduces NK-IS production are under way.

ACKNOWLEDGMENTS

We wish to thank Jacqueline Scott for her excellent technical assistance.

REFERENCES


These sugars are inhibitory when present either throughout the cytotoxicity assay or if their excess is removed by washing (1). These features are comparable to those exhibited by NK-IS, suggesting the latter may represent simple sugars released or shed by nonadherent PCs.

NK-IS may constitute the soluble product of regulatory cells. Nair and Schwartz (19) described a subset of human PBLs that suppresses NK cytotoxicity via a soluble mediator. We failed, however, to detect NK-IS production by human PBLs.  

Cortisol (16) and carrageenan (9) have also been reported to activate suppressor spleen cells (in mice) that regulate NK cytotoxicity by soluble factors. Although we cannot detect NK-IS production by mouse spleen cells, the relevant producer cells may be present in spleen and secrete or shed NK-IS only after activation by cortisol or carrageenan. Suppressor cells activated by cortisone (16) or carrageenan (9) are similar to PCs producing NK-IS in their inability to adhere to plastic, although other characteristics suggest the former belong to the macrophage lineage.

Badger et al. (2) reported the presence of a high-molecular-weight protein (in ascites fluid obtained from patients with ovarian cancer) that inhibits human NK cytotoxicity while having little or no effects on antibody-dependent cell-mediated cytotoxic effectors tested against antibody-coated chick RBCs or HeLa targets. We have also detected material inhibitory to NK cytotoxicity in peritoneal fluid of a patient with ovarian carcinoma.  

NK-IS, by contrast, significantly inhibits antibody-dependent cell-mediated cytotoxic effectors and is a small molecule. Furthermore, it is unclear whether the material in ascitic fluid of cancer patients is the product of PCs or represents circulating proteins that accumulate in peritoneal fluid as the result of changes in vascular permeability.

PCs from C. parvum-injected rats produce less NK-IS than control rats. This correlated with increases in peritoneal NK activity following adjuvant injection. We do not know whether C. parvum directly inhibits NK-IS-producing cells. A likely explanation is that the influx of irrelevant cells into the peritoneal cavity that follows injection reduces the proportion of cells releasing NK-IS. Alternatively, C. parvum may induce the production of mediators that augment NK cytotoxicity and counteract the effects of NK-IS. Brunda et al. (5) reported that C. parvum-activated mouse adherent PCs were less inhibitory of spontaneous or interferon-augmented NK activity than were nonactivated adherent cells. We are uncertain whether the C. parvum effects reported by Brunda et al. (5) and those reported in this manuscript are mediated via the same cellular mechanism. Studies to phenotypically characterize the cells producing NK-IS and the mechanisms whereby C. parvum reduces NK-IS production are under way.

Table 6
Decreased amount of NK-IS produced by PCs from rats given injections of C. Parvum

<table>
<thead>
<tr>
<th>Effector cells</th>
<th>Incubated in</th>
<th>% of specific release at effector:target cell ratio of 25:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat spleen cells</td>
<td>Media</td>
<td>19.2 ± 3a</td>
</tr>
<tr>
<td>NK-IS6</td>
<td>4.2 ± 1</td>
<td></td>
</tr>
<tr>
<td>NK-IS5</td>
<td>11.3 ± 2</td>
<td></td>
</tr>
<tr>
<td>Sera4</td>
<td>17.1 ± 3</td>
<td></td>
</tr>
<tr>
<td>Sera6</td>
<td>18.7 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

a Mean ± S.D. of 3 experiments.

b Rat spleen cells incubated in supernatant from PCs obtained from PBS-injected rats.
c Rat spleen cells incubated in supernatant from PCs obtained from C. parvum-injected rats (1 mg C. parvum i.p. 5 days previously).
d Rat spleen cells incubated in sera obtained from PBS-injected rats.
e Rat spleen cells incubated in sera obtained from C. parvum-injected rats.

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