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Demonstration of T-Cell-Dependent and T-Cell-Independent Components of 8-Mercaptoguanosine-Mediated Adjuvanticity[1] (42126)

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Abstract. The contribution of T cells to potentiation of humoral immunity by the C8-substituted guanine ribonucleosides and the origin of the increased numbers of antigen-responsive B cells generated consequent to their action have been investigated. Augmentation of the antigen-specific antibody response by these nucleosides, exemplified by 8-mercaptoguanosine (8MGuo), can be separated into T-cell-dependent and T-cell-independent components, both by use of the T-cell tropic immunosuppressive agent, cyclosporin A, and by experiments using separated populations of T and B cells. Augmentation of adjuvanticity by T cells is hypothesized to involve a B-cell subpopulation not otherwise subject to the action of 8MGuo. This subpopulation could potentially arise by either of two mechanisms, one whereby preexisting antigen-specific B cells undergo clonal expansion, and one in which cells not normally participating in the response are recruited in the absence of clonal expansion. Although the former mechanism makes a minor contribution to adjuvanticity, the latter mechanism appears to be the dominant one, insofar as models in which 8MGuo-induced proliferation fails to occur (such as after irradiation, or in the SJL mouse) nonetheless exhibit strong adjuvant effects. Analysis of precursor frequency of antigen-specific B cells indicates that for each mature, antigen-responsive B cell present in adult murine spleen, an average of four additional cells can be recruited by the conjoint actions of antigen and 8MGuo. One group subject to such recruitment is the immature antigen-specific B cell, whose degree of functional maturity is accelerated in the presence of antigen and 8MGuo.

Amplification of antigen-specific humoral immune responses by nonspecific adjuvants has been shown to be dependent upon the presence of antigen and upon cellular interactions whose characteristics vary with the particular adjuvant. Recently the potent activity of a novel class of adjuvant, the C8-substituted guanine ribonucleosides, has been described both *in vitro* and *in vivo* (1, 2). This class of adjuvant is unusual because it consists of low mol wt nucleoside analogs that are apparently transported across the plasma membrane to activate B lymphocytes at an intracellular triggering site (3). However, the cellular interactions and mechanisms by which an increased number of antigen-responsive cells is generated have not been rigorously investigated heretofore. The current report describes the role of T cells as well as mechanisms at the level of the B cell, where additional antigen-responsive cells may be produced either (a) endogenously, by a clonal expansion of extant antigen-reactive cells; (b) exogenously, by recruitment of (immature) potentially reactive cells, independently of proliferation; or (c) a combination of (a) and (b). The studies in the present report describe the relevance of each mechanism to the adjuvant activity of the C8-substituted nucleoside 8-mercaptoguanosine (8MGuo).

Materials and Methods. *Mice.* CBA/CaJ and SJL mice, 1–12 weeks of age were purchased from the mouse breeding facility at Scripps Clinic and Research Foundation, La Jolla, California and the Jackson Laboratory, Bar Harbor, Maine. All mice were maintained on Wayne Lab-Blox F6 pellets (Allied Mills, Inc., Chicago, Ill.) and chlorinated water acidified with HCl to a pH of 3.0.

Culture reagents. Constituents of the

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serum-free culture medium employed in these studies have been described elsewhere (4). For serum-containing medium, supportive FCS (Sterile Systems, Inc., Logan, Utah) was substituted for 5% of the volume of RPMI 1640, and Hepes buffer was omitted. 8MGuo was purchased from the Sigma Chemical Company, St. Louis, Missouri.

Cell preparation. Spleen cell and thymocyte suspensions were prepared as described previously (4). B-cell-enriched populations were prepared by injecting mice with 60 μl of rabbit anti-mouse thymocyte serum (ATS) at 3 days and 1 day before sacrifice. Spleen cells (10^8) from these animals were treated with 1:1000 dilution of monoclonal anti-Thy-1.2 (New England Nuclear, Boston, Mass.) for 30 min at 4°C (5). Treated cells were centrifuged at 280*g* for 10 min, antibodies were removed, and the cells were resuspended in a 1:6 dilution of mouse RBC-absorbed guinea pig C at 37°C for 45 min. Cells were then washed and cultured as above. (Adherent cells were depleted by removal of plastic adherent cells twice in succession. This procedure reduced the percentage of esterase-positive cells by approximately 90%.) T-cell-enriched populations were generated by passage over nylon-wool columns (6), a procedure that reduced responsiveness to T-cell mitogens by at least 97%.

Lymphocyte cultures. Murine spleen cells were cultured in microculture plates (No. 3596, Costar, Cambridge, Mass.) at a cell density of 4×10^6 viable cells/ml in a volume of 0.1 ml. Microcultures were incubated at 37°C in a humidified atmosphere of 10% CO_2 in air. Cultures were fed daily with 8 μl of nutritional cocktail. For induction of non-specific immunoglobulin secretion, spleen cells were cultured in 24-well plastic trays (No. 3524, Costar) at a cell density of 5×10^6 viable cells/ml in a volume of 1.0 ml. Culture trays were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

For evaluation of the primary humoral immune response to sheep erythrocytes (SRBC), $4–10 \times 10^6$ murine spleen cells or B cells were cultured in 1.0 ml of 5% FCS-containing medium for 4 days in the presence or absence of antigen. Cells were incubated in culture trays (No. 3524, Costar) at 37°C in a humidified atmosphere of 10% CO_2 in air with the use of tissue culture boxes (CBS Scientific, Del Mar, Calif.) that were rocked at a frequency of seven cycles per minute.

Limiting dilution analysis was carried out by culturing small numbers of cells (10^4–10^5/well) and syngeneic thymocyte filler cells (2×10^5/well) together with 5×10^5 SRBC in the presence or absence of 0.3 m*M* 8MGuo, as adapted from Schreier (7).

Mixed lymphocyte culture (MLC) supernatants. CBA/CaJ spleen cells (10^7) were cultured with 10^7 BDF_1 spleen cells for 4 days in a volume of 5.0 ml in a humidified atmosphere of 10% CO_2 in air at 37°C. Cells were sedimented by centrifugation, and the supernatant medium was subjected to 0.22 filtration before use.

Measurement of DNA synthesis. During the final 24 hr of culture, cells were radiolabeled with 1.0 μCi of [^{3}H]TdR/culture (5 Ci/m*M*, Amersham Radiochemicals, Amersham, England). The microcultures were harvested with a Brandel cell harvester, Model M24V (Biological Research and Development Laboratories, Rockville, Md.) onto glass-fiber filter strips. Filter disks were transferred to plastic scintillation vials, covered with liquid scintillation cocktail, and counted in a Beckman LS-7500 liquid scintillation counter.

Assay of plaque-forming cells (PFC). PFC, secreting antibodies against SRBC, were evaluated after 4 days of culture with a modification of the hemolytic plaque assay of Jerne and Nordin (8).

Immobilization of nucleoside. Bromination of single-stranded poly guanylic acid, average mol wt in excess of 400,000, with a saturated solution of bromine in water was performed by the method of Lafer *et al.* (9). Digests of this material with nuclease P1 and bacterial alkaline phosphatase revealed virtually no unbrominated residues by HPLC analysis on a reverse-phase C18 column. The substituted polymer presents the nucleoside encumbered only at the 3′ and 5′ positions, analogous to that of 8-bromo-cGMP (8BrcGMP), a compound with demonstrated stimulatory activity (10).

Results. In previous studies we have demonstrated that guanine ribonucleosides substituted at C8 with bromine or a sulfhydryl group are potent adjuvants for antibody production to specific antigen both *in vitro* (1)

and *in vivo* (2). Because these substituted nucleosides can provide an alternate source of a T-cell-like differentiative signal for B cells, but also amplify the humoral immune response over and above the level generated in the presence of T cells, the contribution of T cells to the adjuvant effect of 8MGuo was investigated. Using cyclosporin A (CsA) in incremental concentrations to inhibit T-helper-cell activity (11) and the generation of lymphokines (12, 13), it was found that this agent eradicated the underlying response to SRBC entirely (Fig. 1). High concentrations of CsA reduced the nucleoside-amplified response of spleen cell populations to approximately the level of response produced by T-cell-depleted B-cell populations in the presence of antigen and nucleoside. These data suggest the existence of T-cell-dependent as well as independent aspects of immunoenhancement, the former exhibiting sensitivity to CsA, the latter not. The T-independent facet, then, would correspond to the T-cell-like differentiative signals provided by these compounds.

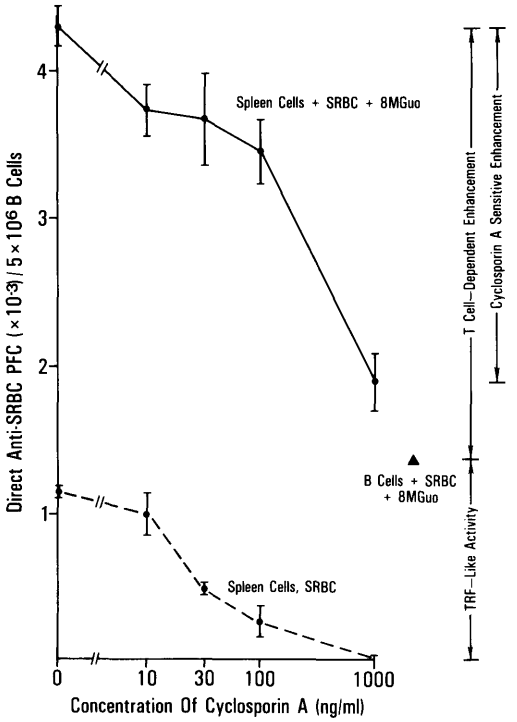

FIG. 1. Delineation of CsA-sensitive and resistant components of adjuvanticity. Viable CBA/CaJ spleen cells or B cells (5×10^6) were cultured in the presence of 2×10^6 SRBC and/or 0.3 m*M* 8MGuo, with incremental concentrations of CsA. Direct PFC to SRBC were evaluated after 4 days. Results are expressed as the arithmetic mean PFC/5×10^6 B cells of triplicate cultures ± SE.

In other experiments the contribution of T cells to adjuvanticity was evaluated directly, using populations of separated splenic B and T cells. Table I demonstrates that addition of T cells to a system containing B cells, antigen, and nucleoside results not in an additive effect but rather in a synergistic one. The degree of synergy in this case correlates well with that shown to be CsA sensitive in Fig. 1.

Having established that enhancement of the primary humoral immune response by 8MGuo is dependent upon the presence of both antigen and nucleoside, the role played by proliferation of antigen-responsive cells (clonal expansion) in adjuvanticity was investigated. During the course of a humoral immune response *in vitro,* clonal expansion is believed to occur primarily during the first 24 hr of culture, followed by receptivity to differentiative signals (14–16). On this basis, the kinetics of adding 8MGuo on different days of culture would suggest that clonal expansion by 8MGuo plays a relatively minor role in immunopotentiation, since delaying the addition of the nucleoside up to 3 days of a 4-day culture period does not appear to impair its ability to transmit a T-cell-like signal or to synergize with T-cell-derived lymphokines (Fig. 2). By this time antigen-induced proliferation has been essentially completed.

A more stringent test of the dependence of adjuvanticity on clonal expansion is to inhibit cellular proliferation with γ irradiation. In previous work, it was found that proliferation was far more sensitive to irradiation than was adjuvanticity (17). Exposure to 150 rad reduced the proliferative response to 8MGuo by more than 92%, and decreased the response to SRBC by about 73%. However, the response to SRBC in the presence of 8MGuo was reduced only by about 30%. Thus, the ratio of responses in irradiated cells cultured with antigen and 8MGuo to those cultured with SRBC alone was equal to or greater than that of unirradiated cells.

TABLE I. CONTRIBUTION OF *T* CELLS TO THE ADJUVANTICITY OF 8MGuo

B cells[a]	T cells	SRBC	8MGuo	Direct Anti-SRBC PFC/culture[b]
+	−	−	−	96 ± 13
+	−	+	−	80 ± 22
−	+	+	−	38 ± 12
+	−	−	+	415 ± 43
+	−	+	+	1158 ± 75
+	+	−	−	146 ± 15
+	+	+	−	310 ± 35
+	+	+	+	4594 ± 194

[a] (5×10^6) Viable CBA/CaJ splenic B cells were cultured with or without 5×10^6 viable syngeneic T cells in the presence of 2×10^6 SRBC and/or 3×10^{-4} *M* 8MGuo in various permutations.

[b] Direct PFC to SRBC were evaluated after 4 days of culture. Results are expressed as the arithmetic mean of two separate experiments ± SE.

Another observation that suggests that adjuvanticity is not absolutely dependent upon clonal expansion stems from the SJL mouse. In this model it is easily observed that although neither proliferative nor polyclonal responses to 8MGuo can be generated (Fig. 3A, B), the nucleoside nonetheless transmits a T-cell-like differentiative signal to B cells from this strain (Fig. 3C). Moreover, when proliferation is assayed under conditions of simultaneous stimulation with antigen and nucleoside, immunoenhancement in the absence of significant proliferation is observed once again (not shown).

Observation of immunoenhancement by 8MGuo in the absence of proliferation (i.e., in the irradiation and SJL models) suggested the possibility that cells not usually participating in the response could be recruited into it by the conjoint actions of antigen and nucleoside. To investigate this possibility, antigen-specific precursor frequency analysis was performed using highly purified B cells cultured with antigen in the presence or absence of 8MGuo. This was done in our usual culture system, so that results would be applicable to it, but with thymocyte filler cells (7). Because these experiments were not performed under maximally stimulatory conditions (i.e., with a combination of such components as 2-ME, bacterial lipopolysaccharide (LPS), dextran sulfate, conditioned medium, Iscove's medium, etc.) the frequencies determined in these experiments are not directly comparable with other, higher values cited in the literature. The results of our studies indicated that whereas one B cell in about 425,000 is responsive to SRBC in this system, one B cell in about 82,000 responds in the presence of nucleoside (Table II). This means that for each antigen-reactive cell in this system, 8MGuo and antigen together recruit an average of 4.2 additional cells that otherwise would have been quiescent.

Earlier observations have suggested that an immature B-cell subpopulation can proliferate in response to 8MGuo (18) and that immunodeficient *xid* mice, lacking pheno-

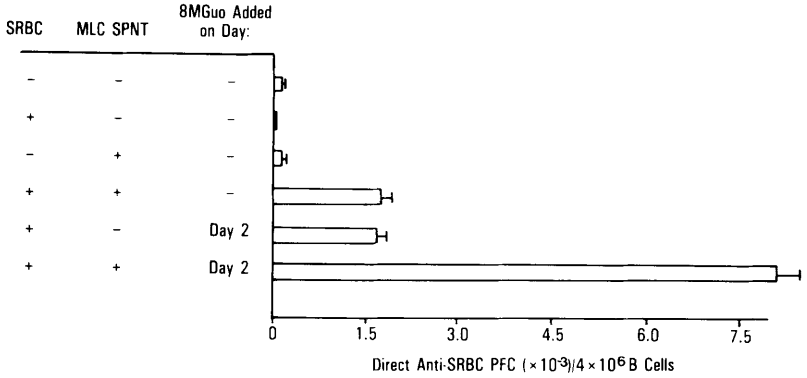

FIG. 2. Effect of late addition of 8MGuo on its T-cell-like effects. Viable CBA/CaJ splenic B cells (4×10^6) were cultured in the presence or absence of 2×10^6 SRBC with or without 10% MLC supernatant. Two days later cultures were supplemented with 1 m*M* 8MGuo as indicated. Direct PFC to SRBC were evaluated 4 days after initiation of culture. Results are expressed as the arithmetic mean of triplicate cultures ± SE.

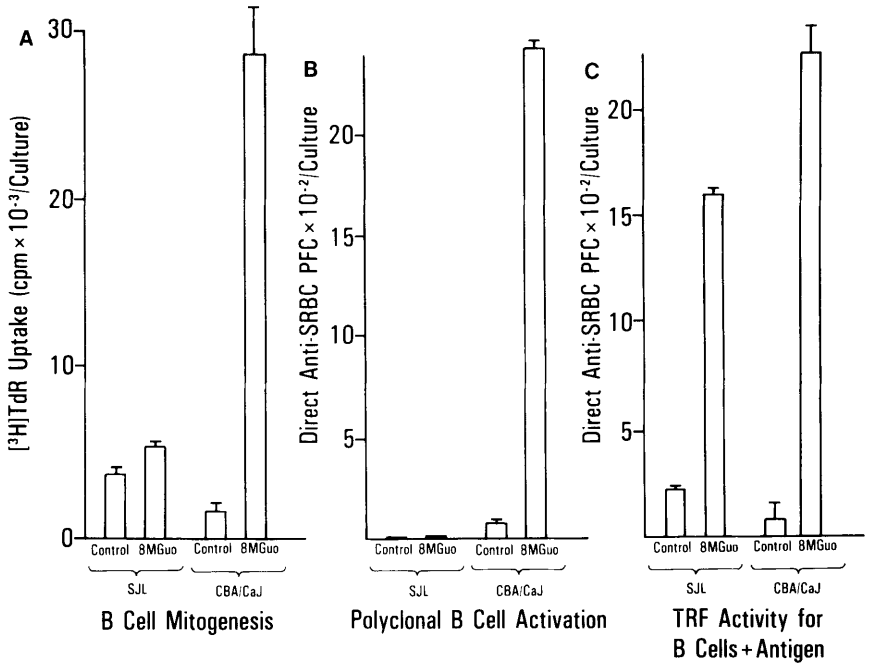

FIG. 3. Dissociation of T-cell-like activity from nonspecific B-cell activation of the SJL mouse. Spleen cells or splenic B cells from either SJL or CBA/CaJ mice were cultured and assayed as described under Materials and Methods for observation of B-cell mitogenesis (A), polyclonal Ig secretion (B), or T-cell-replacing factor activity (C). Results are expressed as the arithmetic mean of five replicate cultures ± SE for A, and the arithmetic mean of triplicate cultures ± SE for B and C.

typically mature B cells, are responsive to the adjuvant effect of 8MGuo (1, 2). Therefore the ability of antigen and 8MGuo to recruit antigen-unresponsive B cells from neonatal mice to respond to antigen was investigated. Neonatal CBA/CaJ mice, 1 week of age, were entirely unresponsive to SRBC (Fig. 4). In the presence of nucleoside, however, these cells generated robust antigen-specific responses, which were of the same magnitude as adult level responses without adjuvant. Thus, it appears that immature B cells constitute at least one group that is recruited by antigen in the presence of 8MGuo.

TABLE II. PRECURSOR FREQUENCY ANALYSIS FOR B CELLS RESPONSIVE TO SRBC IN THE ABSENCE AND PRESENCE OF 8MGuo

	% Nonresponsive cultures when incubated with	
B cells/well[a]	SRBC[b] (%)	SRBC + 8MGuo[b] (%)
2×10^4	95	87.5
4×10^4	92.5	62.5
8×10^4	85	35
10^5	80	27.5
2×10^5	70	N.D.
r^2	0.97[c]	0.96[c]
37% unresponsive	424,260 cells/well	81,860 cells/well

[a] CBA/CaJ B cells were cultured in the numbers shown/well with 4×10^5 syngeneic thymocyte fillers.
[b] Direct PFC to SRBC were determined after 4 days.
[c] r^2 values were calculated by linear regression analysis.

Discussion. The current studies were undertaken to determine the role of T cells in 8MGuo-mediated adjuvanticity and the mechanism by which increased numbers of antigen-responsive B cells are generated in the presence of antigen and 8-mercaptoguanosine. The data obtained suggest that adjuvanticity is constituted of two main components, one T-cell dependent and one T-cell independent. The majority of the increased numbers of antibody-producing cells contributing to the adjuvant effect are re-

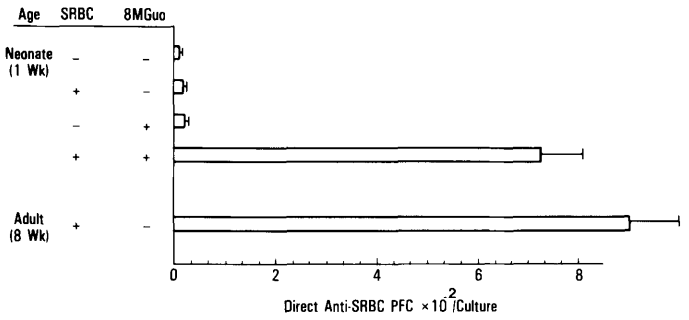

FIG. 4. Reconstitution of the humoral response to SRBC by 8MGuo in neonatal mice. 5×10^6 viable CBA/CaJ spleen cells, taken from mice of various ages as indicated, were cultured in the presence or absence of 2×10^6 SRBC and/or 0.3 m*M* 8MGuo. Four days later direct PFC to SRBC were evaluated. Results are expressed as the arithmetic mean of triplicate cultures ± SE.

cruited from cells not normally participating in the response, with clonal expansion accounting for a minor contribution.

We previously found that populations of murine B cells, purified as rigorously as possible using both *in vivo* and *in vitro* T-cell-depleting strategies, were incapable of responding to T-dependent antigens in a primary (IgM) humoral immune response *in vitro*. Supplementation of these cultures with 8MGuo, however, led to the emergence of an antigen-specific, antigen-dependent immune response, presumably attributable to the nucleoside serving as an alternate T-cell-like signal (19). In the current studies we demonstrate that addition of splenic T cells to such B-cell cultures increases the magnitude of the ensuing response further still, not simply in an additive fashion but rather in a synergistic one. In these studies, as before, the nucleoside itself furnished a somewhat more potent signal for B cells than did T cells. In complementary experiments using cyclosporin A to inhibit the production of lymphokines (12, 13), T-helper activity (11), and possibly responsiveness to lymphokines (13), it was learned that the nucleoside-augmented responses of spleen cells to antigen can be reduced to approximately the level generated by T-cell-depleted populations. In concert these data suggest that the T-cell-like signal transmitted to B cells by 8MGuo differs qualitatively from that provided by T cells. The most probable difference would be in the ability to activate a particular B-cell subpopulation to responsiveness. The subpopulation involved could represent a mature lineage distinct from that which is responsive to antigen alone, or alternately, an immature stage of the population that responds at maturation. The other possible explanation for synergy of 8MGuo and T cells could lie in clonal expansion of antigen-reactive precursor cells by one agent, acting as a B-cell growth factor, and communication of a T-cell-like signal by the other agent, now acting on a considerably enlarged population of antigen-reactive B cells.

Although clonal expansion of antigen-reactive B cells appears to contribute to the ultimate magnitude of the 8MGuo-amplified response, such expansion apparently is not the major mechanism of 8MGuo-induced adjuvanticity. Moreover, it does not appear that proliferation is an absolute requirement for augmentation. Thus, models in which significant proliferation fails to occur (such as after irradiation of lymphoid cells with 150 rad, or the SJL mouse) nonetheless exhibit strong adjuvant effects. Furthermore, the observation that C8-substituted guanosine compounds can be added 2 or 3 days after culture initiation with retention of adjuvanticity (1) is consistent with an effect on an ongoing immune response, well after initial rounds of antigen-induced proliferation are completed (16). However, irradiation consistently reduces the augmented response by approximately 30%. Furthermore, in limiting dilution experiments, it was noted that the average number of cells per positive well was larger in the presence of 8MGuo than in its

absence. These data indicate that clonal expansion does in lesser measure contribute to 8MGuo-mediated immunoenhancement.

The dominant mechanism by which augmentation of antigen-specific responses is achieved, however, is by recruitment of additional, immature antigen-specific B-cell precursors into the response. Recruitment is most clearly reflected in the increased precursor frequency of antigen-specific B cells observed in limiting dilution experiments. For every mature, antigen-responsive B cell present in adult murine spleen, an average of four additional cells can be recruited by the conjoint actions of antigen and 8MGuo. The implication that precursor B cells are the group recruited is consistent with our earlier observation that B cells from CBA/N mice (with the *xid* immunodeficiency), which lack B cells of mature surface phenotype, nonetheless undergo 8MGuo-augmented responses to antigen (1, 2). The possibility that specific precursor B cells are recruited was further tested by examining the immune responsiveness of neonatal mice. These cells, which are entirely unresponsive to SRBC in their own right, exhibited significant antigen-specific responses upon supplementation with 8MGuo; these latter responses reached adult levels by 1 week of age. This further suggested that the nucleoside acts by accelerating the functional maturity of these cells. The data do not address the question of whether these immature cells also develop the surface phenotypic markers characteristic of more mature cells.

The mechanisms of adjuvanticity of other agents have been studied primarily from a cellular perspective. Thus, the roles of T cells and macrophages in humoral immunoenhancement mediated by LPS have been studied by a number of investigators (20–22), whereas the origin of the newly responsive cells has been subject to less intensive study. Quintans and Lefkovits found that in the absence of T cells, antigen-specific B cells undergo clonal expansion only in the presence of both antigen and adjuvant (LPS) (23). Thalhammer *et al.*, examining the effect of LPS and dextran sulfate on the response of porcine lymphocytes to SRBC, found that primary responses were unchanged, whereas subsequent antigenic challenge alone elicited a heightened secondary response suggestive of expansion of memory B cells (24). Prunet and Panijel filtered lymphocytes through antigen-conjugated affinity columns, and found that whereas the primary response to antigen was lost, it could be recovered by addition of LPS to culture (25). This suggested that recruitment of a distinct lymphocyte subpopulation was dependent upon antigen and LPS. Using different approaches, our data substantiates a similar mechanism for the adjuvanticity of 8-mercaptoguanosine.

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