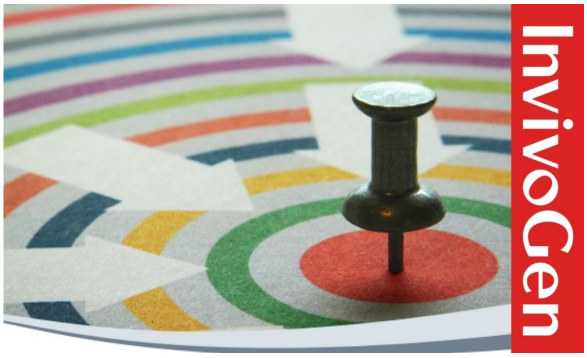


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# SELECTIVE EFFECTS OF CYCLOSPORIN A ON FUNCTIONAL B CELL SUBSETS IN THE MOUSE

ANNALISA KUNKL[1] AND G. G. B. KLAUS

*From the Division of Immunology, National Institute for Medical Research, Mill Hill, London, NW7 1AA, U. K.*

**Cyclosporin A, an immunosuppressive peptide of fungal origin, was believed to selectively affect T lymphocyte functions and to have minimal affects on B lymphocytes. This study shows that, in the mouse, T-dependent B cells and those responding to certain T-independent antigens (so-called TI-1 antigens) are indeed resistant to the drug. However, B cells responsive to other TI antigens (TI-2) are exquisitely sensitive. Thus, doses of the drug that completely abrogate responses to dinitrophenylated (DNP) Ficoll or dextran enhance the response to DNP-lipopolysacchride and have minimal effects on the response to DNP-*Brucella abortus*. Virgin T helper cells are sensitive to the drug, whereas primed T cells are not. Cyclosporin A sensitivity therefore represents a novel marker of functional B cell subsets in the mouse and presumably points to fundamental physiologic differences between such subsets.**

Within recent years considerable evidence has accumulated for the existence of functional B cell subsets in the mouse. In particular, there appear to be separate populations of cells responsive to T cell-dependent (TD)[2] antigens (B2 cells), and T-independent (TI) antigens (B1 cells, References 1–5). In addition, extensive studies using the CBA/N mouse strain have provided evidence for heterogeneity amongst B cells responsive to TI antigens. This mouse carries a sex-linked B cell defect manifested by a total incapacity to respond to a variety of TI antigens, such as dinitrophenyl (DNP) conjugates of Ficoll and dextran (so-called TI-2 antigens), while responding more or less normally to TI-1 antigens such as DNP-lipopolysaccharide (LPS) and DNP-*Brucella abortus* (BA) (5, 6). The factors that determine such functional differences between B cell subsets and the ontogenetic relationships between these subsets are still matters of considerable speculation.

The work described here emerged from studies on the role of T cells in the generation of B memory cells to TD antigens (to be published). In these experiments we used the immunosuppressive drug cyclosporin A (CS-A) in an attempt to selectively inhibit T helper cell function. CS-A is a cyclic undecapeptide produced by the fungi *Trichoderma polysporum* and *Cylindrocarpon lucidum*, which has been used with considerable success in suppressing allograft rejection (reviewed in 7). Its mode of action is unknown, but the available data suggest that it acts preferentially on proliferating T cells, with considerably less effect on B cells (8–10). Subsequent experiments in our study showed that although some B lymphocytes in the mouse are indeed resistant to the drug, a functional subset, namely those responsive to TI-2 antigens, are exquisitely sensitive. CS-A sensitivity therefore affords a novel B cell marker in the mouse.

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[1] Recipient of a Wellcome Trust Fellowship.

[2] Abbreviations used in this paper: BA, *Brucella abortus*; CS-A, cyclosporin A; DEX, B512 dextran; FIC, Ficoll; LPS, *Escherichia coli* lipopolysaccharide; KLH, keyhole limpet hemocyanin; TD, T cell-dependent; TI, T cell-independent.

## MATERIALS AND METHODS

*Animals.* CBA/Ca and (CBA/Ca × C57BL6)$F_1$ mice were bred under specific-pathogen-free conditions at N. I. M. R. In most experiments male mice 9 to 12 wk old were used. Outbred athymic (nu/nu) mice and their nu/+ littermates were obtained from the Breeding Unit of the Imperial Cancer Research Fund, Mill Hill. They were 8 wk old.

*Antigens.* Dinitrophenyl-lysyl-Ficoll (DNP-FIC, containing 31 DNP groups/mol), and DNP-lysyl-B512 dextran (DNP-DEX, m.w. $2 \times 10^6$, containing 5.7 groups/50,000 m.w.) were prepared by CNBr activation as described previously (11). *Escherichia coli* lipopolysaccharide (LPS; 055: B5, Difco, Detroit, MI) was dinitrophenylated in a similar fashion. The preparation contained 2.8 DNP groups/50,000 m.w. and was prepared by Dr. D. W. Dresser; 2,4,6-trinitrophenyl- (TNP) BA was prepared by Dr. S. Marshall-Clarke, by the method of Mond *et al.* (5).

Keyhole limpet hemocyanin (KLH; Calbiochem, La Jolla, CA) was dinitrophenylated using dinitrofluorobenzene. It contained 67 DNP groups/$10^6$ m.w.

*Immunizations.* Mice were given optimal doses of DNP-FIC, DNP-DEX, DNP-LPS, or TNP-BA in phosphate-buffered saline i.p. or i.v., as indicated. For primary responses to DNP-KLH, the soluble antigen was given i.v. in some experiments or i.p. as an alum-precipitate together with $10^9$ *Bordetella pertussis* organisms. Mice were primed for secondary responses by giving 100 μg alum-DNP-KLH plus *B. pertussis* and were used 1 to 3 mo later.

*Immunosuppressive drugs.* CS-A (OL 27-400 N) was kindly provided by Dr. J. F. Borel, Sandoz Ltd., Basel, Switzerland. Since the drug is water insoluble, it was routinely administered dissolved in olive oil. For most experiments a stock solution of 15 mg/ml was prepared by stirring at 60°C until the CS-A dissolved. Mice received 0.07 to 0.1 ml of this solution subcutaneously, to give a dose of 50 mg/kg. Controls received the equivalent volume of olive oil. CS-A is poorly soluble in olive oil at >20 mg/ml. Thus, for experiments employing doses greater than 50 mg/kg, the drug was first dissolved at 500 mg/ml in alcohol and then diluted to 50 mg/ml in olive oil.

Cyclophosphamide (Ward-Blenkinsop, London) was dissolved in saline and given i.p.; 2-amino-6-mercaptopurine (6-

thioguanine, Sigma Chemicals, St. Louis, MO) was suspended at 3 mg/ml in saline at pH 9.6 and was also given i.p.

*Plaque-forming cell (PFC) assays.* Hemolytic PFC specific for DNP were assayed using sheep erythrocytes (SRBC) sensitized with DNP-coupled Fab fragments of rabbit anti-SRBC antibody in Cunningham chambers as described previously (12). In some experiments, background (anti-SRBC) PFC were also assayed. Indirect PFC were developed with a polyvalent rabbit anti-mouse immunoglobulin antiserum, which does not inhibit IgM PFC. IgG PFC are therefore given as indirect PFC minus direct PFC. Most data are presented as arithmetic means ± SE of PFC/spleen.

In some experiments, antibody affinity was estimated by hapten inhibition of PFC (13). Epsilon-DNP-lysine was included in the plaquing mixture to final concentrations ranging from $10^{-4}$ to $10^{-7}$ M in half-log increments. Affinities are expressed as $I_{50}$ values, i.e., molarity of hapten required to inhibit 50% of PFC. The Shannon heterogeneity index (14) was calculated to provide an estimate of heterogeneity of affinity.

## RESULTS

*Differential effects of CS-A on responses to DNP-Ficoll and DNP-LPS.* Borel *et al.* (8) found that daily doses of 200 mg/kg CS-A (*per os*) had no effect on primary serum antibody responses to LPS in athymic (nu/nu) mice, which suggested that CS-A had minimal effects on B lymphocytes. In preliminary experiments, we found that 50 mg/kg CSA given subcutaneously on days −1 to 3 totally abolished the day 4 PFC response to DNP-FIC. To resolve this apparent discrepancy, we compared the effects of various treatments with CS-A on the responses of mice to DNP-FIC and DNP-LPS (Fig. 1). CS-A (50 mg/kg) given on days −1, 0, 1, 2, and 3 caused a 90% suppression of the day 4 IgM response to DNP-FIC and totally abolished the IgG response. Treatment on days −1, 0, and 1 was equally effective, whereas dosing on days −1 and 0 or only on day −1 induced less immunosuppression.

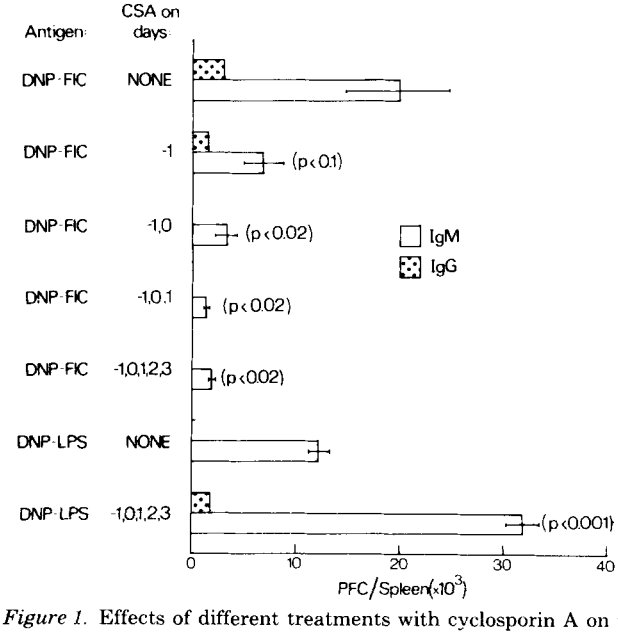


*Figure 1.* Effects of different treatments with cyclosporin A on the responses to DNP-Ficoll and DNP-LPS. (Groups ($n$ = 4) of (CBA × C57BL)$F_1$ mice were given 50 μg DNP-LPS or 20 μg DNP-FIC on day 0, 50 mg/kg CS-A on the days indicated, and were assayed on day 4. Figures in brackets represent probability values for $t$-tests against the appropriate controls (i.e., given olive oil on days −1, 0, 1, 2, and 3).

In striking contrast, 5 days treatment with CS-A produced a 2.5-fold *enhancement* of the response to DNP-LPS. In subsequent experiments, we found that 3 doses (on days −1, 0, and 1) of 2.5 to 5 mg/kg CS-A still produced significant (40%) suppression of the DNP-FIC response (data not shown). Clearly, the response to this antigen is exquisitely sensitive to suppression by CS-A.

A further experiment was done to investigate whether higher doses of the drug would also suppress the response to DNP-LPS (Table I). The results showed that 100 or 150 mg/kg CS-A also produced significant enhancement of the response to this antigen, whereas 300 mg/kg had no effect if the response was expressed as PFC/spleen. However, these mice showed signs of toxicity, and their spleens were substantially smaller than those of controls. Therefore, when the data were expressed as PFC/$10^6$ spleen cells, even 300 mg/kg produced significant enhancement of the response. The response to DNP-LPS is obviously extraordinarily resistant to CS-A.

It should be noted here that daily treatment with 50 mg/kg CS-A never induced any signs of toxicity, nor did it affect the cellularity of the spleen. Furthermore, this dose also had no effect on the polyclonal response to LPS as measured by the number of anti-SRBC PFC in mice given DNP-LPS or by the number of anti-DNP PFC in mice given LPS alone (data not shown).

The results shown in Figure 1 suggest that CS-A is most effective when given around the time of immunization, i.e., that it affects an early stage of lymphocyte triggering (10, 15). The experiment shown in Figure 2 confirms this. Groups of mice were given a single dose of 50 mg/kg CS-A on different days before and after immunization with DNP-FIC. As shown above, this does not induce complete suppression. However, the degree of suppression attained (75 to 80%) was maximal if the drug was given on day −1, 0, or 1, whereas on days 2 to 4 it was no longer effective. It is uncertain whether the enhanced response elicited by treatment on day 3 is meaningful.

We were interested in determining whether CS-A preferentially affects high-affinity precursor cells, i.e., if it in effect promotes tolerance induction. We therefore compared the hapten inhibition profiles of the residual anti-DNP IgM PFC in the group given CS-A on day −1 with those of control mice. The results in Table II show that there was no discernible difference in the relative affinity or heterogeneity of the antibodies produced by the 2 groups.

*Effects of CS-A on responses to other TI-1 and TI-2 antigens.* The results obtained thus far suggested that CS-A might selectively affect responses to TI-2 antigens. To explore this idea,

TABLE I

*Effects of various doses of cyclosporin A on the response to DNP-LPS*

| Group[a] | CS-A | IgM PFC/Spleen | IgM PFC/$10^6$ Cells |
|---|---|---|---|
| | *mg/kg* | | |
| 1 | 100 | 20,400 ± 4,400 (240)[b] | 160 ± 30 (290) |
| 2 | 150 | 30,000 ± 2,700 (355) | N.D.[c] |
| 3 | 300 | 6,400 ± 1,000 (76) | 134 ± 10 (240) |
| 4 | None | 8,500 ± 1,400 (100) | 55 ± 9 (100) |

[a] Groups of (CBA × C57BL)$F_1$ mice were given 50 μg DNP-LPS (day 0), and various doses of CS-A on days −1, 0 and 1; group 4 received olive oil. PFC assay day 4. Student's $t$-tests: PFC/spleen: 1 *vs* 4, $p < 0.05$; 2 *vs* 4, $p < 0.001$; 3 *vs* 4, $p > 0.2$; PFC/$10^6$ cells: 1 *vs* 4, $p < 0.02$; 3 *vs* 4, $p < 0.01$.

[b] Figures in brackets represent percentages of control response.

[c] N.D., not done.

mice were immunized with DNP-FIC or DNP-DEX (TI-2 antigens), or TNP-BA or DNP-LPS (TI-1 antigens), with or without 3 doses of 50 mg/kg CS-A. The results (Fig. 3) again illustrate marked suppression of the response to DNP-FIC (6% of controls). The response to DNP-DEX was also largely abolished (16% of control). However, the response to DNP-LPS was significantly enhanced, whereas that to TNP-BA was only modestly suppressed (60% of control).

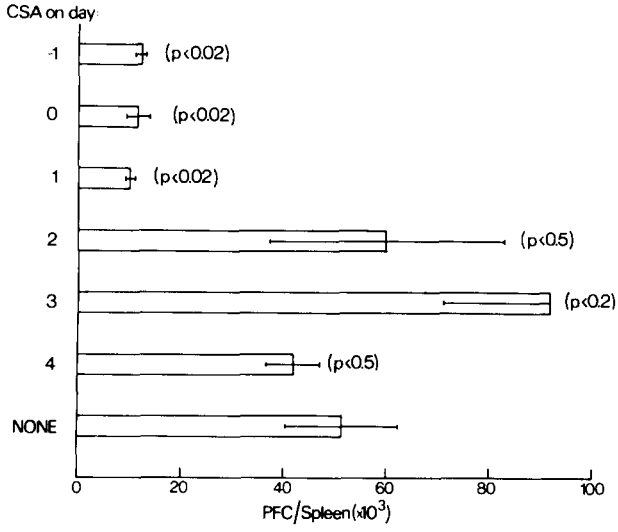


*Figure 2.* Effects of single doses of cyclosporin A given on different days on the response to DNP-Ficoll. Groups of (CBA × C57)$F_1$ mice were immunized with 20 μg DNP-Ficoll on day 0 and given a single dose of CS-A (50 mg/kg) on the day indicated. IgM PFC were assayed on day 5. Probability values were derived by comparison with the control group, given olive oil on day −1.

TABLE II

*Effects of cyclosporin A on the affinity and heterogeneity of the IgM response to DNP-Ficoll*

| Group[a] | CS-A | IgM PFC/spleen | $I_{50} \times 10^{-6}$ M[b] | Heterogeneity Index[c] |
|---|---|---|---|---|
| 1 | + | 12,300 ± 1,200 | 2.76 ± 0.18 | 1.96 ± 0.57 |
| 2 | − | 51,700 ± 9,700 | 3.24 ± 0.54 | 2.12 ± 0.04 |

[a] Groups of (CBA × C57BL)$F_1$ mice received 20 μg DNP-FIC; group 1 received 50 mg/kg CS-A on day −1, and group 2 received olive oil. PFC assay day 5.

[b] Concentration of ε-DNP-lysine required to inhibit 50% of PFC.

[c] Shannon heterogeneity index (14). This provides a measure of heterogeneity of affinity of the response. Under the assay conditions used the index ranges from 0 (homogeneous response) to 2.58 (highly heterogeneous).

These results therefore support the concept that responses to TI-2 antigens are much more susceptible to CS-A than those to TI-1 antigens. However, it has become clear that responses to TNP-BA and DNP-LPS differ in susceptibility. Thus, 50 mg/kg CS-A induced modest (but significant) suppression of the response in TNP-BA in 2 out of 3 experiments, whereas it routinely enhanced the response to DNP-LPS 2- to 3-fold.

*Effects of CS-A in nude mice.* To establish whether these results reflected direct effects of the drug on B cells responding to TI-1 and TI-2 antigens, groups of nu/nu and nu/+ mice were given CS-A and immunized with DNP-FIC or DNP-LPS (Table III). The response to DNP-FIC was markedly reduced in both athymic and euthymic mice. In experiment A, CS-A enhanced the response of nu/+ mice to DNP-LPS but had no effect on the response in nu/nu mice. In the second experiment, only 1 nu/nu mouse given DNP-LPS and CS-A survived, so it is still uncertain whether the enhancement of the response to this antigen is a TD phenomenon.

*Effects of other immunosuppressants on responses to DNP-FIC and DNP-LPS.* We subsequently considered the possibility that B cells responsive to TI-2 antigens might be inherently more susceptible to any immunosuppressive agent. We therefore compared the effects of cyclophosphamide and 6-thioguanine on responses to DNP-FIC and DNP-LPS (Table IV). In experiment A, mice were given various doses of cyclophosphamide at the time of immunization. Forty milligrams per kilogram caused 90% suppression of the responses to both antigens, whereas lower doses had progressively less effect. There was no discernible difference in the susceptibility of the responses to the 2 antigens.

In experiment B, mice were given various doses of 6-thioguanine 1 day after immunization (the optimal time for the action of this drug; see 16). The responses to DNP-FIC and DNP-LPS were both quite susceptible to suppression by this drug. However, again there was no real difference in the susceptibility of the responses elicited by these 2 antigens.

These results strongly suggest that the B cells responsive to TI-2 antigens are only hypersusceptible to CS-A and not to more traditional immunosuppressants.

*Effects of CS-A on TD antibody responses.* Having demonstrated the existence of CS-A-sensitive and resistant TI B cell populations, it was obviously important to study the susceptibility of TD B cells to the drug. In an initial experiment mice

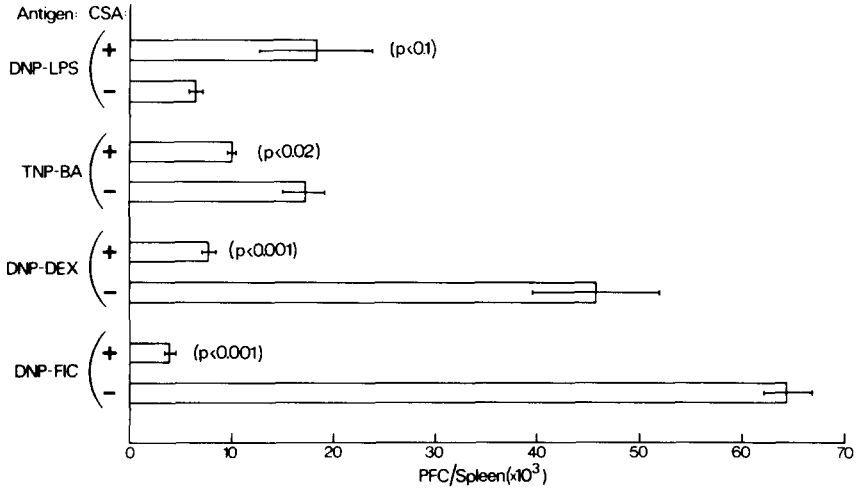


*Figure 3.* Effects of cyclosporin A on responses to TI-1 antigens (DNP-LPS and TNP-BA) or TI-2 antigens (DNP-DEX and DNP-FIC). (CBA × C57)$F_1$ mice were given TNP-BA ($6 \times 10^8$ organisms i.v.), DNP-LPS (50 μg i.p.), DNP-FIC (20 μg i.p.) or DNP-DEX (150 μg i.p.) on day 0, and 50 mg/kg CS-A s.c. on days 1, 0, and 1. IgM PFC were assayed on day 5. Probability values were derived from *t*-tests with the appropriate control given olive oil.

TABLE III

*Effects of cyclosporin A on responses to DNP-Ficoll and DNP-LPS in nu/nu and nu/+ mice*

| Expt. | Antigen | CS-A | nu/nu[a] | | nu/+ | |
|---|---|---|---|---|---|---|
| | | | IgM PFC/spleen[b] | IgG PFC/spleen | IgM PFC/spleen | IgG PFC/spleen |
| A | DNP-FIC | − | 20,300 ± 4,500 | 7,400 ± 4,900 | 72,200 ± 18,300 | 4,000 ± 3,300 |
| | DNP-FIC | + | 5,000 ± 1,900 | 30 ± 30 | 6,200 ± 2,500 | 2,400 ± 780 |
| | DNP-LPS | − | 3,800 ± 1,500 | 1,100 ± 1,100 | 2,900 ± 500 | 1,200 ± 700 |
| | DNP-LPS | + | 4,000 ± 600 | | 11,600 ± 1,500 | 3,900 ± 1,900 |
| B | DNP-FIC | − | 48,800 ± 4,400 | 24,800 ± 7,800 | 28,900 ± 5,500 | 15,900 ± 4,400 |
| | DNP-FIC | + | 7,900 ± 1,900 | 3,700 ± 700 | 3,400 ± 2,000 | 1,200 ± 600 |
| | DNP-LPS | − | 9,600 ± 2,600 | 5,200 ± 3,400 | 5,200 ± 300 | 24,000 ± 9,600 |
| | DNP-LPS | + | 16,300[c] | 3,900 | 15,500 ± 2,000 | 27,500 ± 6,400 |

[a] Groups (n = 3) of outbred nu/nu or nu/+ mice given 20 μg DNP-LPS or DNP-FIC (day 0) and 50 mg/kg CS-A on days −1, 0, and 1; PFC assay on day 4 (Expt. A) or day 5 (Expt. B).

[b] Student's *t*-tests (IgM response). Expt. A: nu/nu DNP-FIC, $p < 0.05$; DNP-LPS, $p > 0.5$; nu/+ DNP-FIC, $p < 0.05$; DNP-LPS, $p < 0.01$. Expt. B: nu/nu DNP-FIC, $p < 0.01$; nu/+ DNP-FIC, $p < 0.02$; DNP-LPS, $p < 0.01$.

[c] One surviving mouse.

TABLE IV

*Effects of cyclophosphamide or 6-thioguanine on responses to DNP-Ficoll and DNP-LPS*[a]

| Expt. | Drug Dose | IgM PFC/Spleen to DNP-FIC[a] | | IgM PFC/Spleen to DNP-LPS | |
|---|---|---|---|---|---|
| | *mg/kg* | | | | |
| A (cyclophosphamide)[b] | 40 | 4,300 ± 1,700 | (11) | 3,200 ± 1,000 | (13) |
| | 20 | 17,800 ± 7,300 | (45) | 7,300 ± 500 | (30) |
| | 10 | 31,300 ± 7,800 | (79) | 18,000 ± 1,000 | (73) |
| | 5 | 19,600 ± 5,500 | (50) | 22,300 ± 8,400 | (91) |
| | 0 | 39,600 ± 6,100 | (100) | 24,600 ± 2,400 | (100) |
| B (6-thioguanine)[c] | 20 | 250 ± 100 | (2) | 450 ± 90 | (6) |
| | 10 | 1,600 ± 700 | (10) | 1,100 ± 300 | (14) |
| | 5 | 3,900 ± 500 | (23) | 2,800 ± 600 | (37) |
| | 2.5 | 8,600 ± 300 | (51) | 3,000 ± 500 | (41) |
| | 0 | 16,800 ± 1,600 | (100) | 7,500 ± 700 | (100) |

[a] Groups of CBA (Expt. A) or (CBA × C57BL)$F_1$ (Expt. B) mice given 50 μg DNP-LPS or 20 μg DNP-FIC on day 0. PFC assay, day 4.

[b] Cyclophosphamide given at the time of immunization.

[c] 6-Thioguanine given on day 1. Figures in brackets represent percent of control responses.

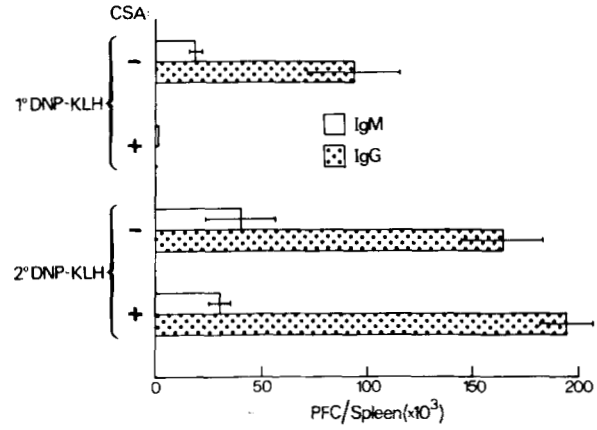


*Figure 4.* Effects of cyclosporin A on primary and secondary responses to DNP-KLH. For the primary (CBA × C57)$F_1$ mice were given 100 μg alum-precipitated DNP-KLH plus *B. pertussis* on day 0, with or without 50 mg/kg CS-A on days −1 through 6; they were assayed on day 7. For the secondary response, mice primed as above 2 months previously were boosted (day 0) with 20 μg soluble DNP-KLH, treated with 50 mg/kg CS-A on days −1 through 3, and assayed on day 4.

were given either a primary injection of alum-precipitated DNP-KLH plus *B. pertussis* or were boosted with soluble DNP-KLH 2 mo after the priming dose (Fig. 4). Treatment with 50 mg/kg CS-A on days −1 to 6 totally ablated the primary response to DNP-KLH (day 7 assay). In contrast, this dose had no effect on the secondary response (day 4 assay).

These data indicate that primed (secondary) TD B cells and primed T helper cells are resistant to this dose of CS-A. The inhibition of the primary response, however, could reflect inactivation of primary B cells and/or T helper cells. To distinguish between these possibilities, mice primed 4 wk previously with KLH (which should have CS-A-resistant T helper cells) were immunized with DNP-KLH. We used soluble DNP-KLH in this experiment, since previous trials had revealed that challenging KLH-primed mice with alum-precipitated DNP-KLH could result in suppression of the anti-DNP response.

The results of this experiment (Table V) showed that 3 days' treatment with CS-A caused significant suppression of the peak IgM (day 3) and IgG (day 5) responses in mice not preimmunized with KLH. In contrast, the drug had no effect on the day 3 IgM response or on the day 5 IgG response in KLH-primed mice. Interestingly, the day 5 IgM (but not IgG) response in these mice was somewhat enhanced by CS-A. The reproducibility of this phenomenon is currently under study.

These results indicate that primary TD B cells, like secondary TD B cells, and B cells responsive to TI-1 antigens are CS-A resistant. Furthermore, primary T helper cells appear to be CS-A sensitive, unlike primed helper cells.

## DISCUSSION

The major conclusion from this study is that murine B lymphocytes can be broadly divided into 2 compartments, CS-A sensitive and CS-A resistant, and that these compartments correlate with functional subsets defined by other criteria. It thus appears that TD B cells (both primary and secondary) and those responsive to TI-1 antigens are resistant, whereas those responding to TI-2 antigens, i.e., the subset lacking in the young adult CBA/N mouse (6, 17) are exquisitely sensitive. In addition, virgin T helper cells appear to be CS-A sensitive, whereas primed T cells are not (Fig. 4, Table V). The effects of CS-A on T cells merit further study, but these results agree with previous data on the efficacy of the drug in suppressing a variety of T cell-mediated primary responses (7, 8, 15).

Such striking disparities in drug sensitivity between different lymphocyte subsets must point to fundamental physiologic differences between CS-A-sensitive and -resistant compart-

TABLE V

*Effects of cyclosporin A on primary responses to DNP-KLH in normal vs KLH-primed mice*[a]

| Group | Mice[b] | CS-A[c] | PFC/Spleen on | | | |
|---|---|---|---|---|---|---|
| | | | Day 3 | | Day 5 | |
| | | | IgM | IgG | IgM | IgG |
| 1 | Normal | − | 14,900 ± 3,300 | 7,800 ± 1,100 | 7,400 ± 2,000 | 36,100 ± 12,100 |
| 2 | Normal | + | 3,600 ± 900 | 2,400 ± 200 | 5,100 ± 700 | 4,400 ± 1,100 |
| 3 | KLH-primed | − | 48,600 ± 7,900 | 10,900 ± 5,100 | 9,400 ± 1,700 | 59,000 ± 18,700 |
| 4 | KLH-primed | + | 44,600 ± 11,800 | 10,100 ± 1,900 | 42,500 ± 18,500 | 55,600 ± 12,300 |

[a] Student's *t*-tests: Day 3, 1 *vs* 2 IgM, $p < 0.02$, IgG $p < 0.01$; 3 *vs* 4 IgM, $p > 0.5$, IgG, $p > 0.5$. Day 5, 1 *vs* 2 IgM, $p > 0.3$, IgG $p < 0.05$; 3 *vs* 4 IgM, $p > 0.1$, IgG $p > 0.5$.

[b] (CBA × C57BL)$F_1$ mice: KLH-primed mice had received 100 μg alum-precipitated KLH plus *B. pertussis* 6 weeks previously. All were immunized (day 0) with 100 μg soluble DNP-KLH given i.v.

[c] 50 mg/kg CS-A given on days −1, 0, and 1; controls received olive oil.

ments, either at the time of antigen recognition or in the immediate events after triggering. CS-A evidently acts at an early stage in lymphocyte activation and has no discernible effects on unstimulated cells (10). Unfortunately, little is known of its mode of action except that in mitogen-stimulated lymphocytes, CS-A rapidly inhibits the uptake of uridine, thymidine, and amino acids (18). Since CS-A is highly lipophilic, it is reasonable to assume that it would bind to, and perhaps traverse, cell membranes with great facility. There is yet no indication for any selective binding of the drug to different lymphocyte populations (D. J. G. White, personal communication). It is, however, still possible that CS-A sensitivity does reflect subtle differences in cell membrane properties. The CS-A-sensitive (DNP-FIC-responsive) B cell subset is known to carry the antigens Lyb3 and Lyb5, and to bear more surface IgD than IgM (19–21), but whether these markers have any relevance to the susceptibility of these cells to CS-A clearly requires further study.

A second possibility is that CS-A, like many immunosuppressive drugs, acts at a specific stage in the cell cycle, e.g., in $G_0$, with less effect in $G_1$ or later. If so, this would imply that CS-A-resistant cells have already been preactivated, perhaps shifted from a pre-progenitor to a progenitor compartment by nonspecific stimuli as suggested by the work of Shortman *et al.* (reviewed in 22). There is at present no evidence for this, but this hypothesis is amenable to study.

It seems unlikely that CS-A facilitates tolerance induction by TI-2 antigens, as do drugs such as cyclophosphamide (23). If this were so, one might expect the residual antibody response in partially immunosuppressed mice to be of lower affinity, which is not the case (Table II). Indeed, the susceptibility of TI-2 responses to immunosuppression seems to be unique to CS-A, since neither cyclophosphamide (an alkylating agent) nor 6-thioguanine (a purine analogue related to azathioprine) caused selective suppression of the response to DNP-FIC (Table IV). *In vitro* experiments by Galanaud *et al.* (24) indicated that TD B cells are highly sensitive to azathioprine, whereas those responding to the TI antigen TNP-phage T4 are not. Since it is uncertain whether TNP-T4 is a TI-1 or a TI-2 antigen (25), the relevance of these results to our own is unclear.

The findings that DNP-LPS-responsive B cells and TD B cells, are CS-A resistant fits into the concept that DNP-LPS stimulates TD B cells, but in a T-independent fashion (4, 25–27). This simple picture is, however, clouded by the data of Tittle and Rittenberg (25) showing that DNP-LPS stimulates both TD and TI (DNP-FIC-responsive) B cells to give secondary *in vitro* IgG responses. Since such responses can only be obtained using lymphocytes from hyperimmunized mice, the relevance of this finding to the situation *in vivo* remains to be established.

Our results with TNP-BA suggest that it differs immunogenically from DNP-LPS, since 50 mg/kg CS-A, which routinely enhanced the response to DNP-LPS, caused about 50% suppression of the response to TNP-BA (Fig. 3). In agreement with this idea, Quintans and McKearn (28) found differences in tolerance susceptibility between B cells responsive to TNP-BA and DNP-LPS. Perhaps the cells responsive to TNP-BA are in a transitional stage between the CS-A-sensitive and the CS-A-resistant compartments, or perhaps TNP-BA stimulates cells in both compartments. These results certainly suggest that a simple division of TI antigens into TI-1 and TI-2 groups on the basis of the responsiveness of the CBA/N mouse is an oversimplification. The properties that determine the capacity of an antigen to stimulate B cells in this mouse are still obscure. Two TI-1 antigens (LPS and *Nocardia* mitogen) are clearly B cell mitogens, although BA is apparently a very weak mitogen (5). It is generally accepted that TI-2 antigens, such as Ficoll and dextran, are not polyclonal B cell activators.

The enhancement of the response to DNP-LPS by CS-A is a highly reproducible phenomenon, the significance of which is unclear. It remains to be seen whether this effect also occurs with other antigens, using different treatments with the drug. Thus far, it has not been observed with DNP-FIC, although the response to this antigen is still suppressed by much lower doses of CS-A than we routinely used. It is an attractive possibility that the enhancement of the DNP-LPS response reflects an effect of CS-A on the cell membrane, since both the drug and the antigen are so lipophilic.

CS-A sensitivity therefore clearly represents a novel B cell marker in the mouse, although it should be mentioned that little is known about possible effects of the drug on T cell subsets. We are currently studying the duration of suppression of TI-2 responses by CS-A. If the drug causes functional elimination of the responding clones of cells, then CS-A would be a powerful tool for analyzing lymphocyte subpopulations. Little information exists on this point. It is clear that short courses of CS-A induce marked prolongation of organ graft survival, far outlasting the persistence of the drug (7). This does not, however, appear to hold for skin grafts, which are rejected soon after the cessation of drug therapy (15, 29).

Finally, it is difficult to decide whether the present findings have any clinical relevance. If human T and B cells respond similarly to the drug as do those of the mouse, one would predict that CS-A would have little impact on ongoing antibody responses and so would be of limited use for the suppression of autoantibody production. Furthermore CS-A should inhibit some primary antibody responses but leave others (to certain bacterial infections?) unaffected. One may safely conclude, how-

ever, that CS-A represents a major advance in the search for agents with selective immunosuppressive activity.

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