Proc. Natl. Acad. Sci. USA
Vol. 77, No. 3, pp. 1593–1596, March 1980
Immunology

Nucleic acid-specific suppressor T cells

(immunosuppression/autoantigens/autoimmunity)

YVES BOREL AND MICHAEL C. YOUNG

Division of Immunology, Children's Hospital Medical Center and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115

Communicated by Richard L. Sidman, December 13, 1979

ABSTRACT **The concept of using cell-bound antigens as tolerogen was applied to nucleic acid. Nucleoside was linked directly to spleen cell suspensions. Intravenous administration of nucleoside coupled to isogeneic spleen cells into mice generated suppressor cells that diminished the formation of antibody-forming cells either to a T-dependent antigen *in vivo* or to a T-independent antigen *in vitro*. Suppressor cells were nucleoside specific, but the specificity of immune suppression seems to be somewhat broader than that of tolerance to a single nucleoside. The ability to raise nucleic acid-specific suppressor T cells may have implications for both the pathogenesis and treatment of systemic lupus erythematosus.**

The modification of lymphoid cell surfaces by haptens (1, 2) has been used to elicit hapten-specific suppressor T cells (3) or cytotoxic T cells (4). This principle could be applied to autoantigens, such as nucleic acids. A means of inducing suppressor T cells specific for nucleic acid antigens would have particular significance for systemic lupus erythematosus (5–7), a disease in which tissue damage is mainly due to complexes of anti-nucleic acid antibodies with nucleic acid antigens (8, 9). We have already shown that the induction of immunologic tolerance to nucleic acids by nucleosides conjugated to isologous IgG impaired the development of lupus nephritis in (NZB × NZW)F_1 mice (10, 11). Here we show that nucleoside coupled to isogeneic spleen cells elicits suppressor T cells that are nucleoside specific.

MATERIALS AND METHODS

Animals. Six- to eight-week-old male C57BL/6 or (C57BL/6 × DBA/$_2$)F_1 (referred to as BDF_1) mice were purchased from Jackson Laboratory.

Preparation of Antigens and Tolerogen. The nucleosides adenosine (A), guanosine (G), cytosine (C), and thymine ribonucleoside (T) were purchased from Sigma. Keyhole limpet haemocyanin (KLH) (Pacific Biomarine, Venice, CA) was prepared as described (12). Murine IgG_{2a} was obtained through starch block electrophoresis from sera of BALB/c mice bearing the RPC5 tumor, as described (12). Aminoethylcarboxymethyl-Ficoll (AECM-Ficoll) was prepared according to Inman (13). G was linked to KLH, IgG_{2a}, or AECM-Ficoll, and all four nucleosides (A, G, C, and T, termed AGCT) were linked to KLH by the method of Erlanger and Beiser (14). The following hapten-protein conjugates were used: G_{176}-KLH (G-KLH), G_{35}-IgG_{2a} (G-IgG), G_{58}-AECM-Ficoll (G-Ficoll), and $AGCT_{212}$-KLH (AGCT-KLH). In each case, subscripts indicate the total molar ratio of hapten substitution on the carrier protein.

G or AGCT were coupled to spleen cells by the method previously described (14) but slightly modified to prevent lymphoid cell lysis. The procedure was as follows: spleen cells from 10 C57BL/6 mice were prepared in 15 ml of minimal Eagle's medium by using a tissue grinder. The cells were washed three times in minimal Eagle's medium and once in 50 ml of a 50% solution of the Eagle's medium and 0.15 M $NaHCO_3$ at pH 8; the cells were resuspended in 2 ml of 50% minimal Eagle's medium/$NaHCO_3$ solution and cell concentration was determined in a hemocytometer. For every 10^9 spleen cells, 20 mg of G or 20 mg of AGCT (5 mg of each nucleoside) was used. Twenty milligrams of G or 20 mg of AGCT was suspended in 3 ml of 0.15 M $NaHCO_3$, then oxidized with 1.5 ml 0.1 M sodium periodate in saline for 20 min at room temperature; the reaction was stopped with 15 μl of ethylene glycol. The spleen cell suspensions were added dropwise to the oxidized nucleoside solutions and the mixture was stirred gently at room temperature. After 15 min the binding was stopped with 100 mg of *t*-butylamine borane in 5 ml 0.15 M $NaHCO_3$. After 3 min at room temperature, the reaction tube was filled to 50 ml with minimal Eagle's medium and centrifuged for 15 min at 175 × *g*. The G- or AGCT-conjugated cells were washed with minimal Eagle's medium three more times, after which appropriate adjustments in cell concentration were made. G- or AGCT-coupled cells were then ready for intravenous injection via tail vein. Sham-treated cells were prepared in an identical manner except for the addition of 1.5 ml of sodium periodate solution without nucleoside. Subsequent treatment with ethylene glycol and *t*-butylamine borane and washings were unchanged.

Detection of Antibody-Forming Cells. Nucleoside-specific plaque-forming cells were revealed as described (15).

Separation of T Cells. Thymus-derived lymphocytes were separated from spleen cell suspensions after passage through a nylon wool column as described by Julius *et al.* (16).

***In Vitro* Culture.** The Marbrook–Diener method (17) was used to culture spleen cell suspensions *in vitro*.

Statistical Analysis. Experimental results were analyzed according to Student's *t* test.

RESULTS AND DISCUSSION

In the initial experiments, only guanosine was used. This nucleoside is immunodominant in the immune response to the four nucleosides of DNA (adenosine, thymine ribonucleoside, cytosine, and guanosine) (18). Preliminary experiments were done in C57BL/6 mice to determine the dose of G-coupled isogeneic spleen cells that suppresses the immune response to G. Groups

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: AGCT, a mixture of the four nucleosides adenosine, guanosine, cytosine, and thymine ribonucleoside; G-AECM-Ficoll or G-Ficoll, guanosine-aminoethylcarboxymethyl-Ficoll; KLH, keyhole limpet haemocyanin; PFC, plaque-forming cells; SRBC, sheep erythrocytes.

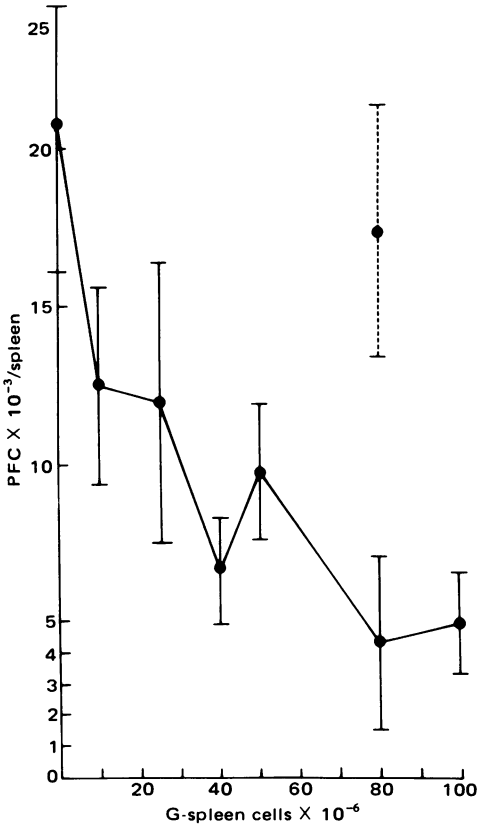

FIG. 1. The dose–response of suppression of the immune response to G-KLH induced by intravenous injection of G-modified spleen cells. Groups of C57BL/6 male mice, ages 6–8 weeks, were injected with various doses of G-spleen cells. Control groups received either no injection or 80 × 10^6 sham-treated cells. All mice were immunized intraperitoneally 5 days later with 0.2 mg of G-KLH in complete Freund's adjuvant. On day 10, individual spleen cell suspensions were made of each spleen; these were assayed for direct plaque-forming cell (PFC) activity to G-conjugated sheep erythrocytes (SRBC). Each point represents the geometric mean PFC per spleen (±SEM) of each group of five mice. The broken bar represents the SEM of the group of five mice injected intravenously with 80 × 10^6 sham-treated spleen cells.

of C57BL/6 mice were injected intravenously with various doses of G-coupled spleen cells. Untreated animals or animals similarly injected with sham-treated spleen cells served as controls. Five days later, all animals were immunized intraperitoneally with G-KLH in complete Freund's adjuvant (18). Suppression of the immune response to G as detected by direct plaque-forming cells was dose dependent (Fig. 1). The optimal dose was 80 × 10^6 of G-coupled isogeneic spleen cells; 100 × 10^6 such cells caused no further suppression. In contrast, intravenous injection of 80 × 10^6 sham-treated cells failed to influence the immune response to G.

The time course of suppression was then determined by varying the day of immunization with G-KLH in relation to the injection of 80 × 10^6 G-coupled spleen cells. We found that the suppression required time to develop and was transient. For example, if immunization with G-KLH was done on the same day as the intravenous injection of G-coupled spleen cells, there was no diminution in the number of anti-guanosine PFC. In contrast, when the immunization was done 2 days after treatment with 80 × 10^6 G-coupled spleen cells, the number of G-specific antibody-forming cells was reduced by 50%. Maximum suppression (85%) was observed when the immunization followed the treatment by 5 days. When the immunization was given 7 days after the treatment, the immune response was reduced by 45%; but if the delay was 14 days there was no effect on the immune response.

Table 1. Specific suppression of guanosine immune response to a T-independent antigen *in vitro*

Treatment	PFC/culture ± SEM G-Ficoll	*P**	SRBC
None	550 ± 176		5688 ± 1216
80 × 10^6 G-spleen cells intravenous	56 ± 129	<0.05	5557 ± 1004
None	558 ± 165		
T cells from animals injected intravenously with 80 × 10^6 G-spleen cells mixed *in vitro* 1:1 with normal spleen cells†	56 ± 26	<0.05	

* *P*, probability that difference in response between treated and control cells is due to chance.

† The immune response to G-Ficoll of normal T cells mixed *in vitro* 1:1 with normal spleen cells was 593 ± 121.

The transient nature of this phenomenon is consistent with the results of others in different systems (19, 20) and suggests that we are dealing with suppressor T cells. To substantiate this interpretation, we determined whether spleen cell suspensions obtained from animals injected intravenously with G-coupled spleen cells 5 days previously could suppress the B cell response to the T-independent antigen G-AECM-Ficoll *in vitro*. The results (Table 1) show that such is the case. Similarly, nonadherent T cells obtained by passing spleen cell suspensions from suppressed animals through a nylon wool column were able to diminish the immune response of normal spleen cells. The spleen cell suspensions that diminished the response to G-

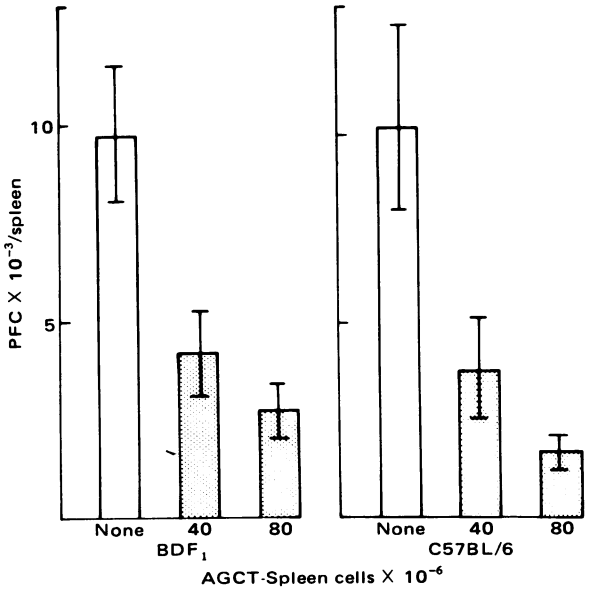

FIG. 2. Comparative suppression of the immune response to AGCT-KLH induced by isogeneic AGCT-modified spleen cells in C57BL/6 and BDF_1 mice. Groups of five mice of either C57BL/6 or BDF_1 strain were given intravenous injections of 40 × 10^6 and 80 × 10^6 isogeneic AGCT-modified spleen cells. All mice (including untreated controls) were immunized intraperitoneally 5 days later with AGCT-KLH in complete Freund's adjuvant. Individual spleen cell suspensions were assayed on day 10 for direct PFC to AGCT-SRBC targets. Values are geometric means ± SEM.

Table 2. Nucleoside specificity of suppression *in vivo*

Group	Treatment†	PFC/spleen ± SEM AGCT-SRBC	P*	A-SRBC	P*	G-SRBC	P*	C-SRBC	P*	T-SRBC	P*
I	None	21,824 ± 8382		6428 ± 2160		18,808 ± 8908		10,108 ± 3744		8502 ± 3309	
II	Sham-treated spleen cells‡	20,328 ± 4272		6009 ± 1514		17,178 ± 4657		7,757 ± 1892		8555 ± 2497	
III	G-IgG_{2a}	11,454 ± 2250	<0.05	4569 ± 1172	<0.2	4,821 ± 2893	<0.01	9,943 ± 2306	<0.5	7853 ± 2968	<0.5
IV	G-spleen cells‡	11,501 ± 2849	<0.05	2868 ± 713	<0.005	5,789 ± 1797	<0.025	4,484 ± 790	<0.005	6844 ± 1482	<0.2

* *P*, probability that difference in response between treated and control (group 1) animals is due to chance.
† Administered 5 days before immunization.
‡ There were 80×10^6 of the appropriately treated spleen cells.

AECM-Ficoll did not affect the immune response to SRBC *in vitro*, suggesting that the suppression was antigen specific.

The above data suggest that suppressor T cells to guanosine reduce either the T-dependent immune response *in vivo* or the T-independent response *in vitro*. We next examined whether an immune response to the four nucleosides AGCT can be suppressed *in vivo*. Groups of C57BL/6 or BDF_1 mice were injected intravenously with either 40×10^6 or 80×10^6 isogeneic spleen cells coupled to AGCT. Five days later, these mice, together with untreated mice of the same strain, were challenged with the four nucleosides bound to KLH (AGCT-KLH). Fig. 2 shows that the immune response to the nucleosides was suppressed in both strains by either dose of AGCT-coupled spleen cells.

In view of the potential therapeutic application of this model to the treatment of systemic lupus, we asked if G-coupled spleen cells could inhibit an immune response to the four nucleosides. For comparison, we included C57BL/6 mice made tolerant by a single intravenous injection of G coupled to mouse IgG, because the hapten specificity of tolerance induced by isologous IgG is known (15). In all instances, antibody-forming cells to either AGCT or to the individual nucleosides were assayed in the same spleen cell suspension. We also examined the immune response to SRBC in animals treated with G-coupled spleen cells to further study the specificity of the system. The results of these experiments (Table 2) show that treatment with G-coupled spleen cells appears to affect not only the immune response to the ligand present on the modified lymphoid cell (i.e., G), but also the immune responses to A and C. In contrast, as expected, tolerance induced by isologous IgG was G specific. (In both instances, the number of PFC revealed by AGCT-SRBC targets was reduced due to the immunodominant role of G.) Thus, immune suppression induced by G-coupled spleen cells appears to have a specificity that is somewhat broader than tolerance to G induced by isologous IgG. The suppression was, however, specific for nucleosides, because there was no effect on the immune response to an unrelated antigen, SRBC, *in vivo* (PFC to SRBC = 158,700 ± 30,200 in animals treated with 80×10^6 G-coupled spleen cells 5 days before challenge with 0.1 ml of 20% SRBC suspension; cf. 158,600 ± 21,500 in untreated animals similarly challenged).

We have shown that the concept of using cell-bound antigens as tolerogens applies to nucleic acids. It should be emphasized that immune suppression generated by nucleoside-modified lymphoid cells suppresses not only the immune response to a T-dependent antigen *in vivo* but also the immune response to a T-independent antigen *in vitro*. This suggests that suppressor T cells affect either helper T cells or B cells directly.

Several areas of investigation appear to be opened by the demonstration of nucleoside-dependent suppressor T cells. For example, not only suppressor T cells but also T cells cytotoxic for nucleoside might be elicited by this technique, thus providing a model for the study of cell-mediated immunity to nucleic acid antigens, an unexplored field. Second, the role played by the major histocompatibility antigen on the carrier lymphoid cell of the donor animal in eliciting either suppressor or cytotoxic T cells can be examined in a model relevant to autoimmune diseases. Third, the finding that the specificity of immune suppression induced by G-coupled IgG is somewhat broader than the specificity of tolerance induced by G-coupled IgG is perhaps of greatest biological importance in determining the therapeutic value of raising nucleic acid-specific suppressor T cells to treat murine systemic lupus. Perhaps the specificity of the suppressor T cell product is broader than that of the immunoglobulin B cell receptor. Conceivably, oligonucleotide linked to cell surfaces might suppress antibodies to either single- or double-stranded DNA, which are of critical importance in lupus nephritis. In any event, it appears that the suppression of antibody to nucleic acid antigens can now be achieved by two different means: either through inducing tolerance with nucleic acid antigen linked to gamma globulin or by eliciting suppression with nucleic acid linked to cells. The relative contribution of either or both applications of immunologic tolerance can be assessed for treating an autoimmune disease.

We thank James Bangs and Lynne Kilham for expert assistance and Dr. B. D. Stollar for providing us with the G_{58}-AECM-Ficoll. This work was supported by National Institutes of Health Grants AI 13867 and AM 16392, and in part by the Lupus Foundation of Massachusetts.

1. Battisto, J. R. & Bloom, B. R. (1966) *Nature (London)* **212**, 156–157.
2. Asherson, G. L. & Zembala, M. (1974) *Proc. R. Soc. London Ser. B:* **187**, 329–348.
3. Miller, S. D. & Claman, H. N. (1976) *J. Immunol.* **117**, 1519–1526.
4. Shaerer, G. M. (1974) *Eur. J. Immunol.* **4**, 527–533.
5. Allison, A. C., Denman, A. M. & Barnes, R. D. (1971) *Lancet* ii, 135–140.
6. Talal, N. & Steinberg, A. D. (1974) *Curr. Top. Microbiol. Immunol.* **64**, 79–103.
7. Krakauer, R. S., Waldman, T. A. & Strober, W. (1976) *J. Exp. Med.* **144**, 662.
8. Köffler, D., Schur, P. H. & Kunkel, H. B. (1967) *J. Exp. Med.* **126**, 607–623.
9. Lambert, P. H. & Dixon, F. J. (1968) *J. Exp. Med.* **127**, 507–521.
10. Borel, Y., Lewis, R. M. & Stollar, B. D. (1973) *Science* **182**, 76–78.
11. Borel, Y., Lewis, R. M., André-Schwartz, J., Stollar, B. D. & Diener, E. (1978) *J. Clin. Invest.* **61**, 276–282.

12. Borel, Y., Golan, D. T., Kilham, L. & Borel, H. (1976) *J. Immunol.* **116,** 854–858.
13. Inman, J. K. (1975) *J. Immunol.* **114,** 704–709.
14. Erlanger, B. F. & Beiser, S. M. (1964) *Proc. Natl. Acad. Sci. USA* **52,** 68–74.
15. Stollar, B. D. & Borel, Y. (1977) *Nature (London)* **267,** 158–160.
16. Julius, M. H., Simpson, E. & Herzenberg, L. A. (1973) *Eur. J. Immunol.* **3,** 645–649.
17. Diener, E. & Armstrong, W. D. (1967) *Lancet* **i,** 1281–1283.
18. Stollar, B. D. & Borel, Y. (1976) *J. Immunol.* **117,** 1308–1313.
19. Scott, D. W. (1978) *Cell. Immunol.* **37,** 327–335.
20. Moody, C. E., Innes, J. B., Siskind, G. W. & Weksler, M. (1978) *J. Immunol.* **120,** 844–849.