CLINICAL IMMUNOLOGY AND IMMUNOPATHOLOGY 45, 366–374 (1987)

An Immunomodulating Anti-rheumatic Drug, Lobenzarit Disodium (CCA): Inhibition of Polyclonal B-Cell Activation and Prevention of Autoimmune Disease in MRL/Mp-*lpr/lpr* Mice

MASAHIKO MIHARA, TOSHIAKI NAKANO, AND YOSHIYUKI OHSUGI[1]

Section of Immunopharmacology, Research Laboratories, Chugai Pharmaceutical Co., Ltd., 41-8, Takada 3-chome, Toshimaku, Tokyo 171, Japan

In this study, we examined the effect of an immunoregulatory antirheumatic agent, lobenzarit disodium (CCA), on spontaneously developing glomerulonephritis in MRL/Mp-*lpr/lpr* (MRL/*l*) mice. Starting from 6 weeks of age, mice were given CCA orally 5 days a week at a dose of 2 or 10 mg/kg. A control group was given the same volume of distilled water. The CCA treatment suppressed the excretion of protein in the urine. At 40 weeks of age, the incidence of proteinuria was 10/10 in the controls, 6/10 in the 2-mg/kg treatment group, and 5/10 in the 10 mg/kg group. The life span was prolonged dose dependently. The 50% survival time was 33 weeks for the controls, 35.5 weeks for the 2-mg/kg group, and 41 weeks for the 10-mg/kg group. The serum levels of anti-ssDNA antibody, anti-TNP antibody, and rheumatoid factor (RF) of the Ig G isotype and immune complex were reduced compared with control group. But the antibodies of Ig M isotype were not reduced. The serum Ig G1, Ig G2, and Ig G3 were significantly lower in the CCA-treated mice than in the controls. But again the serum level of Ig M was unchanged. These effects of CCA may be based on the suppression of lymphadenopathy. CCA may correct abnormal B-cell growth and differentiation factor release by the MRL/*l* abnormal T cells. These results show that CCA inhibits the development of lupus nephritis in MRL/*l* mice through the amelioration of the abnormal immune response, polyclonal B-cell activation.

INTRODUCTION

Lobenzarit disodium (CCA; disodium 4-chloro-2,2'-iminodibenzoate) is a novel antirheumatic agent which is therapeutically effective in rheumatoid arthritis (1) through its immunoregulating activity. It is noteworthy that CCA has no anti-inflammatory effect in spite of its structural similarity to mefenamic acid, a potent nonsteroidal anti-inflammatory agent (2).

Our previous studies have shown that CCA has a therapeutic effect on adjuvant arthritis in rats (3) and on autoimmune glomerulonephritis spontaneously developed in NZB/W F1 mice (4). The immunomodulating activity has also been demonstrated in several experimental systems in mice. CCA activates the function of suppressor T and/or helper T cells, depending on the magnitude of the immune response, and improves abnormal antibody production (5). In this context, CCA is quite different from the so-called immunosuppressive drugs such as alkylating agents, antimetabolites, and so on.

MRL/Mp-*lpr/lpr* (MRL/*l*) mice spontaneously and aggressively develop an au-

[1] To whom correspondence should be addressed.

0090-1229/87 $1.50

toimmune disease, which resembles human systemic lupus erythematosus (SLE). It is characterized by autoantibody production, immune complex (IC) glomerulonephritis, and death from renal failure (6). In addition, MRL/*l* mice can be used as an arthritis model, since these mice have rheumatoid factor (RF) in the circulating blood (7).

Abe *et al.* (8) have reported that in MRL/*l* mice, CCA inhibits the development of autoimmune disease and reduces IC deposition in the renal glomeruli and articular synovium. However, the mechanisms of the CCA action have not been determined.

In this study, we examined in MRL/*l* mice the effect of CCA treatment on the serum levels of autoantibodies, antihapten antibody and IC, and on each class or subclass of immunoglobulins to see whether CCA ameliorates the immunological abnormality, polyclonal B-cell activation.

MATERIALS AND METHODS

Animals. The MRL/*l* mice used were from a colony maintained at the Laboratory Animal Center of our Research Laboratories by propagation from breeding pairs supplied by Dr. Murphy of the Jackson Laboratory (Bar Harbor, ME). The animals were specific pathogen free and were kept in cages in a room maintained at 24 ± 2°C and 50–60% relative humidity.

Drug administration and experimental schedule. CCA (Chugai Pharmaceutical Co., Ltd., Tokyo) was dissolved in distilled water and administered orally to male mice. The CCA was given 5 days a week beginning at 6 weeks of age and continuing until death. The control group received comparable volumes of distilled water. Blood was collected at 8, 17, 20, and 24 weeks of age and assayed for anti-TNP antibody, anti-ssDNA antibody, RF, and IC. Proteinuria was measured every 2 weeks from the eighth week. In a similar experiment separately performed, female mice were given CCA starting from 5 weeks of age and were sacrificed at 17 weeks of age to measure the weight of the lymph node and spleen.

Proteinuria. This was determined semiquantitatively by means of Combistix paper (Sankyo Co., Ltd., Tokyo) impregnated with tetrabromophenol blue at pH 3.0. A grading of 0 to 4 was made on the basis of the protein concentration in the urine, the five categories being respectively 0–30, 30–100, 100–300, 300–1000, and >1000 mg/dl.

Serum anti-TNP antibody. The serum anti-TNP antibody level was measured by enzyme-linked immunosorbent assay (ELISA) on microtiter plates (Nunc-Immuno plate 1, Roskilde, Denmark). Mouse serum albumin (MSA; Sigma Chemical Co., St. Louis, MO), previously trinitrophenylated (TNP-MSA; 50 μg/ml), was pipetted in volumes of 100 μl into wells of a microtiter plate and incubated for binding at 37°C for 2 hr. The plate was then thoroughly rinsed, dilutions of test serum were added to the wells in volumes of 100 μl, and then the plates were incubated for another 2 hr at room temperature. After washing the wells, 100 μl of a 4000-fold dilution of peroxidase-conjugated anti-mouse Ig M or anti-mouse Ig G antibody (Cappel Laboratory) was added to each well, and the plate was reincubated at room temperature for 2 hr. The wells were then rinsed and 100 μl of *o*-phenylene diamine solution (0.4 mg/ml) was added to each well. After 15–30

min at room temperature, 50 μl of 6 N H_2SO_4 was added to each well to arrest enzyme action and the absorbance (O.D.; 492–540 nm) was measured with a Titertek Multiskan (Flow Laboratories Inc., McLean, VA).

Serum anti-ssDNA antibody. Serum anti-single-stranded DNA (anti-ssDNA) antibody was also measured by ELISA. Denatured DNA prepared by boiling a solution of calf thymus DNA (50 μg/ml; Type 1, Sigma Chemical Co.) with subsequent rapid cooling was bound to the wells of a microtiter plate by incubation with 100 μl added to each well. The plate was then washed, and 100 μl of serial dilutions of test serum was pipetted into the wells. After reincubation at room temperature for 2 hr, the plate was rinsed and the assay was carried out in the same manner as that described for anti-TNP antibody assay.

Serum rheumatoid factor (RF). To determine Ig G RF and Ig M RF, microtiter plates were incubated to bind goat Ig G (10 μg/ml) or mouse Ig G (10 μg/ml) (Cappel Laboratory) with 100 μl in each well. After rinsing, 100 μl of serial dilutions of test serum was pipetted into the wells and incubated at room temperature for 2 hr, followed by the same procedure as described for the anti-TNP antibody assay.

Immune complex(IC). IC was measured according to the method of Creighton *et al.* (9). Briefly, 25 μl of mouse serum was added to 600 μl of borate buffer solution (0.1 M, pH 8.4), and then 625 μl of 6% polyethylene glycol solution was added (final concentration of 3% PEG 6000, Wako Pure Chemical Industries, Ltd., Osaka, Japan). After 18 hr of incubation at 4°C, the precipitated IC was obtained and redissolved. The protein content was measured using a Bio-Rad protein assay kit (BIO-RAD Chemical Division, Richmond, VA).

Serum immunoglobulin levels. Serum levels of Ig M, Ig G1, Ig G2, and Ig G3 were determined by single radial immunodiffusion, as previously reported (10, 11). Antibodies against mouse Ig M, Ig G1, and Ig G2 were purchased from Meloy Laboratories (Springfield, MA) and Ig G3 was from Litton Bionetics (Charleston, SC). Immunoglobulin reference standards were also commercially available (Meloy Laboratories and Litton Bionetics).

Statistical data analysis. Statistical significance of difference was analyzed by Student's t test.

RESULTS

Appearance of Proteinuria

In the control group, proteinuria began to appear at 18 weeks of age and all mice were positive at 40 weeks of age. In contrast, CCA-treated mice excreted less protein; it decreased dose dependently. At 40 weeks of age, the incidence of proteinuria was 60 or 50% in the groups of mice treated respectively with 2 or 10 mg/kg of CCA (Fig. 1).

Life Span

CCA prolonged the life of male MRL/*l* mice in a dose-dependent manner (Fig. 2). The 50% mortality time for the controls was 33 weeks, and 90% of mice were dead by 41 weeks of age. But CCA treatment prevented fatal renal disease and the 50% mortality time for the both CCA-administered groups was 41 weeks.

FIG. 1. Cumulative percentage of mice with positive proteinuria in male MRL/l mice treated with CCA. Proteinuria over grade 2 was assessed as positive. Each group was consisted of 10 mice. (●) Control; (○) CCA, 2 mg/kg; (△) CCA, 10 mg/kg.

Serum Anti-ssDNA Antibody Levels

There was an age-related marked increase of both the Ig M and Ig G classes of anti-ssDNA antibody in mice both treated and untreated. However, CCA at doses of 2 or 10 mg/kg equally suppressed the serum levels of the anti-ssDNA antibody of the Ig G class, compared to untreated controls (Fig. 3). The Ig M class of anti-ssDNA antibody was slightly suppressed by the administration of 10 mg/kg of CCA, compared with the controls.

Serum RF Levels

Serum RF reached a maximum at 20 weeks of age (Fig. 4). CCA treatment significantly suppressed the Ig G class of RF in a dose-dependent manner, but the Ig M class of RF was not reduced.

Serum Anti-TNP Antibody Levels

In the controls, serum anti-TNP levels of both Ig G and Ig M classes increased with age (Fig. 5). The increase of the Ig G class was particularly striking. Treatment with CCA suppressed the increase in levels of serum anti-TNP antibody of the Ig G class in a dose-dependent manner, compared with the controls, but did not decrease the Ig M class of anti-TNP antibody.

FIG. 2. Survival curve of male MRL/l mice treated with CCA. The same animals as those in Fig. 1 were used. The symbols are the same as in Fig. 1.

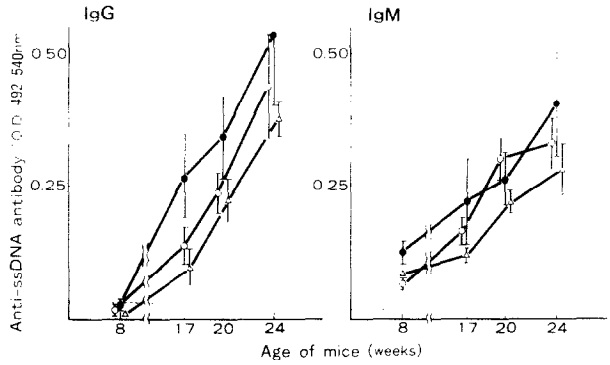

FIG. 3. The development of anti-ssDNA antibodies with age. The points and vertical lines indicate the means and SE of 10 mice (9 mice for the 2 mg/kg CCA-treated group at 24 weeks). The broken line indicates the value of the negative control (C3H/HeN mice). The symbols are the same as Fig. 1.

Serum Levels of IC

The serum IC increased with age. There was a decrease in IC levels in CCA-treated mice ($P < 0.05$ at 17 weeks of age at 10 mg/kg of CCA) (Fig. 6).

Immunoglobulin Levels

In the CCA-treated mice there was a significant decrease in levels of Ig G1, Ig G2, and Ig G3, but not in Ig M (Fig. 7).

Body Weight

There was no difference in the body weight gains among the control and CCA-treated groups (Table 1).

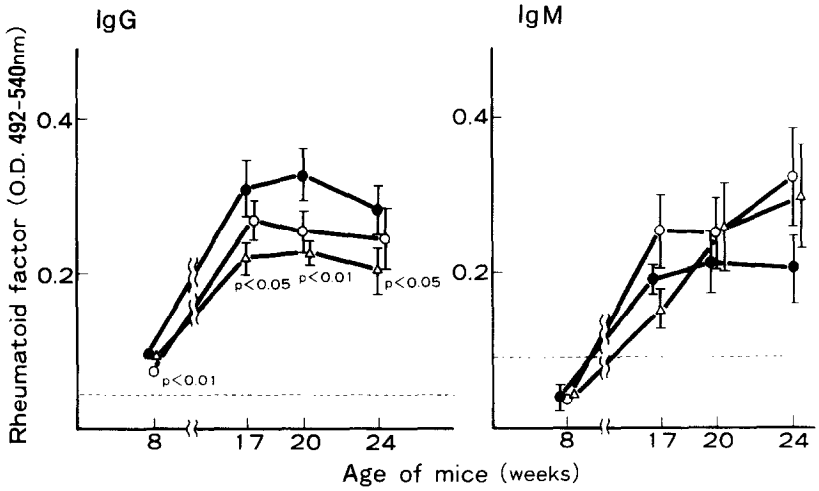

FIG. 4. The development of rheumatoid factors with age. (See legend for Fig. 3).

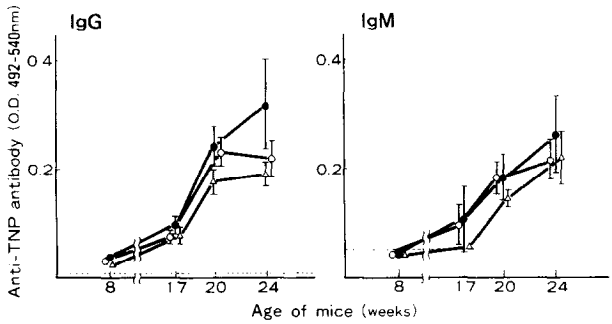

FIG. 5. The development of anti-TNP antibodies with age. (See legend for Fig. 3.)

Weight of Lymph Node and Spleen

Both the mesenteric lymph nodes and the spleens lost weight in CCA-treated mice (mean ± SE, mg) (Table 2).

DISCUSSION

These results indicate that CCA effectively inhibits the development of spontaneously occurring autoimmune syndrome in MRL/*l* mice. Treatment with CCA from 6 weeks of age markedly prolonged the survival time and suppressed the excretion of protein into the urine in these mice.

NZB/W F1 female mice, MRL/*l* mice, and BXSB male mice are murine models (lupus mouse) of human systemic lupus erythematosus (12). From the results of the many experiments using these autoimmune mice, Dixon *et al.* (13) have classified them into two groups based on the mechanisms of pathogenesis of the immunological abnormalities. One group, called B-lupus mice, includes NZB/W F1 female and BXSB male mice. In these mice the B lymphocytes are hyperresponsive to stimuli which induce B-cell activation, that is, I-BCDF, lipopolysaccharide (LPS), and so on. Mice in the other group, represented by the MRL/*l* strain, respond almost normally to the B lymphocyte activators, but their abnormal T

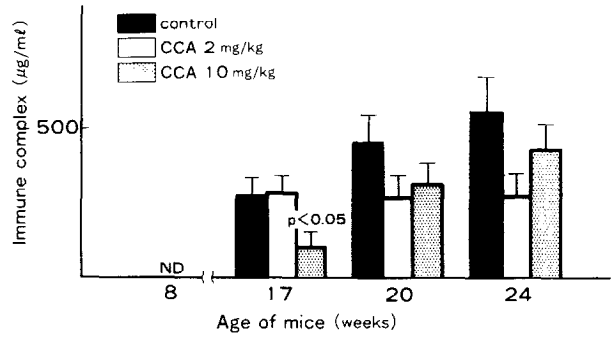

FIG. 6. Serum levels of the immune complex in male MRL/*l* mice treated with CCA. The columns and vertical lines indicate the means and SE of 10 mice (9 mice for the 2 mg/kg CCA-treated group at 24 weeks). ND, not detected.

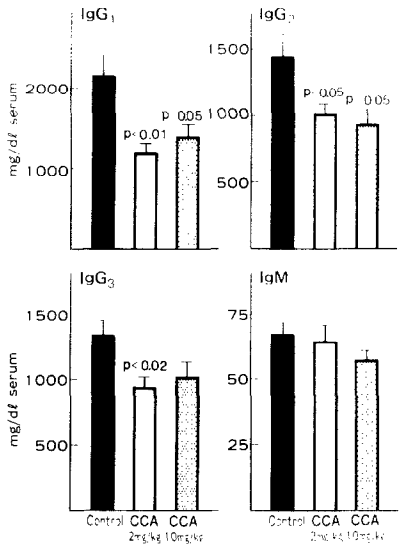

FIG. 7. Serum immunoglobulin levels at 20 weeks of age. (See legend for Fig. 6.)

lymphocytes release a factor which induces B-cell activation. Nevertheless, it has been proved that the common immunological abnormality in all of these mice is the polyclonal B-cell activation (PBA) (14), which also gives rise to autoantibody production. Attention has been focused upon the relationship between PBA and development of lupus nephritis. Although polyclonally activated B lymphocytes produce many kinds of immunoglobulins, it is thought that the Ig G class of immunoglobulins plays the greatest role in the development of lupus nephritis, as described below.

Hang *et al.* (15) reported that chronic injections of endotoxin from *Salmonella minnesota* strain Re 595 (LPS Re 595) causes severe glomerulonephritis in NZW, female BXSB, and MRL/*n* mice, but not in the normal strain. In this experiment, antibody of the Ig G class was easily produced in those three mouse strains but not in normal mice.

In addition, the molecules of IC containing the Ig M class antibody are so large that it is easily eliminated from circulating blood by phagocytes. On the other

TABLE 1
BODY WEIGHT GAINS IN MALE MRL/*l* MICE TREATED WITH CCA

Treatment		Age of mice (weeks)			
		8	16	20	24
Control		35.4 ± 0.80[a]	42.9 ± 0.90	43.2 ± 0.92	44.3 ± 0.84
CCA	2	35.2 ± 0.82	42.8 ± 0.59	42.4 ± 1.24	44.9 ± 0.72
(mg/kg)	10	35.2 ± 0.54	42.3 ± 0.60	42.8 ± 0.61	43.9 ± 0.73

[a] Each value indicates mean and SE (g) of 10 mice (9 mice for the CCA 2 mg/kg-treated group at 24 weeks).

TABLE 2
WEIGHT OF LYMPH NODE AND SPLEEN IN FEMALE MRL/l MICE AT 17 WEEKS OF AGE

Treatment	N	Lymph node	Spleen
Control	5	1718.4 ± 164.60	699.2 ± 127.55
CCA 10 mg/kg	7	936.4 ± 69.65	511.4 ± 60.83
		$P < 0.001$	

hand, the molecular size of IC consisting of the Ig G class antibody and antigen is smaller than that of the Ig M class antibody and antigen, so it is scarcely excreted from the circulating blood by phagocytes, but is deposited on renal glomeruli (16).

As shown in Figs. 3–5, 7, CCA preferentially suppressed the production of Ig G antibody, but had no effect on Ig M antibody production relative to any of the parameters of PBA. Moreover CCA inhibited the formation of IC in the circulating blood (Fig. 6). These findings indicate that by suppressing Ig G class antibody-producing PBA, CCA prevents the development of autoimmune nephritis by reducing IC formation which decreases the probability of the IC being deposited in renal glomeruli.

The mechanisms of the suppressive effect of CCA on PBA are not well known. MRL/*l* mice have a single autosomal recessive lymphoproliferative (*lpr*) gene which is responsible for severe autoimmunity in the mice (6). Massively generated lymphocytes, whose cell surface marker revealed dull Lyt-1 + 2 − and L3T4 − along with Ly-5 (B-220) which is a marker antigen present on B lymphocytes (17), spontaneously release a factor named I-BCDF that induces PBA (18). CCA inhibited the generation of these abnormal T lymphocytes and decreased the weight of the lymph node and the spleen. From these lines of evidence, CCA seems to exert a suppressive effect on PBA by inhibiting the generation of these abnormal T lymphocytes.

Although it is not yet known how CCA suppresses the generation of these abnormal T cells, the following findings support the idea: (i) in nude mice, CCA administration causes the pre-T lymphocytes to differentiate into Thy-1-positive cells (19, 20); (ii) adding CCA to pre-T lymphocytes cultures augments T-cell colony formation (Y. Wakabayashi *et al.*, personal communication).

In fact, recently it is reported that these abnormal MRL/*l* T lymphocytes, which produce hardly an interleukin 2 (IL-2), can be caused to differentiate into IL-2-producing T lymphocytes when costimulated with 12-*O*-tetradecanoylphorbol-2-acetate and calcium ionophore A-23187 (21, 22). Further, Budd *et al.* (17) reported that by using a combination of phorbol myristate acetate and IL-2, these abnormal T lymphocytes acquired transient expression of IL-2 receptors and grew in an IL-2-dependent manner, and these proliferating cells underwent differentiation to more mature T lymphocyte phenotype, with loss of cell surface B-220 and acquisition of antigen receptor and Lyt-2.

From these facts, it appears that the T-cell differentiation effects of CCA may be associated with the decrease of abnormal T cells.

There are several papers showing that immunosuppressive agents such as cyclophosphamide, prostaglandin E, anti-Thy-1.2 antibody, and irradiation inhibit massive lymphadenopathy and autoimmune disease (23–26). However, CCA is not an immunosuppressant, as reported previously (2, 27). Thus it is obvious that

the effect of CCA is based on a mechanism distinct from that of the earlier drugs or treatment.

Izui *et al.* (28) reported that SLE-prone mice given a low-calory diet did not produce autoantibodies, so the subsequent nephritis was less severe and survival was prolonged. However the body weight gains in CCA-treated MRL/*l* mice were almost identical to those of the control mice. This indicates that the action of CCA on immune function in MRL/*l* mice is not calory-dependent.

REFERENCES

1. Shiokawa, Y., Horiuchi, Y., Mizushima, Y., Kageyama, T., Shichikawa, K., Ofuji, T., Honma, M., Yoshizawa, H., Abe, C., and Ogawa, N., *J. Rheumatol.* **11**, 615, 1984.
2. Ohsugi, Y., Hata, S., Tanemura, M., Nakano, T., Matsuno, T., Takagaki, Y., Nishii, Y., and Shindo, M., *J. Pharm. Pharmacol.* **29**, 636, 1977.
3. Ohsugi, Y., Nakano, T., and Hata, S., *Immunopharmacology* **6**, 15, 1983.
4. Ohsugi, Y., Nakano, T., Hata, S., Niki, R., Matsuno, T., Nishii, Y., and Takagaki, Y., *J. Pharm. Pharmacol.* **30**, 126, 1978.
5. Yamamoto, I., Ohmori, H., and Sasano, M., Japan. *J. Pharmacol.* **33**, 859, 1983.
6. Murphy, E. D., and Roths, J. B., *Mouse News Lett.* **58**, 51, 1978.
7. Hang, L., Theofilopoulous, A. N., and Dixon, F. J., *J. Exp. Med.* **155**, 1690, 1982.
8. Abe, C., Shiokawa, Y., Hata, S., and Takagaki, T., *The Ryumachi* **21**, 165, 1981.
9. Creighton, W. D., Lambert, P. H., and Miescher, P. A., *J. Immunol.* **111**, 1219, 1973.
10. Mancini, G., Carbonara, A. O., and Heremans, J. F., *Immunochemistry* **2**, 235, 1965.
11. Ohsugi, Y., Gershwin, M. E., Ahmed, A., Skwlly, R. R., and Milich, D. R., *J. Immunol.* **128**, 2220, 1982.
12. Andrews, B. S., Eisenberg, R. A., Theofilopoulos, A. N., Izui, S., Wilson, C. B., McConahey, P. J., Murthy, E. D., Roths, J. B., and Dixon, F. J., *J. Exp. Med.* **148**, 1198, 1978.
13. Dixon, F. J., Theofilopoulos, A. N., McConahey, P., and Prud'homme, G. J., *In* "Progress in Immunology" (Y. Yamamura and T. Tada, Eds.), p. 1115. Academic Press, Tokyo, 1983.
14. Izui, S., McConahey, P. J., and Dixon, F. J., *J Immunol.* **121**, 2213, 1978.
15. Hang, L. M., Slack, J. H., Amundson, C., Izui, S., Theofilopoulos, A. N., and Dixon, F. J., *J. Exp. Med.* **157**, 894, 1983.
16. Finbloom, D. S., and Plotz, P. H., *J. Immunol.* **123**, 1594, 1979.
17. Budd, R. C., MacDonald, H. R., Lowenthal, J. W., Davignon, J., Izui, S., and Cerottini, J., *J. Immunol.* **135**, 3704, 1985.
18. Prud'homme, G. J., Park, C. L., Fieser, T. M., Kofler, R., Dixon, F. J., and Theofilopoulos, A. N., *J. Exp. Med.* **157**, 730, 1983.
19. Minoda, M., Ueda, M., and Horiuchi, A., *Igaku-no-ayumi* **125**, 176, 1983.
20. Nakano, T., Ohsugi, Y., Tanemura, M., Sato, N., Hata, S., and Takagaki, Y., *Japan. J. Inflammation* **3**, 68, 1983.
21. Katagiri, T., Tomiyama, H., Fyuwa, S., and Kano, K., *Int. Arch. Allergy Appl. Immunol.*, **83**, 167, 1987.
22. Katagiri, K., Katagiri, T., Eisenberg, R. A., Ting, J., and Cohen, P. L., *J. Immunol.* **138**, 149, 1987.
23. Shiraki, M., Fujiwara, M., and Tomura, S., *Clin. Exp. Immunol.* **55**, 333, 1984.
24. Kelley, V. E., Winkelstein, A., Izui, S., and Dixon, F. J., *Clin. Immunol. Immunopathol.* **21**, 190, 1981.
25. Seaman, W. E., Wofsy, D. W., Greenspan, J. S., and Ledbetter, J. A., *J. Immunol.* **130**, 1713, 1983.
26. Theofilopoulos, A. N., Balderas, R., Shawler, D. L., Izui, S., Kotzin, B. L., Strober, S., and Dixon, F. J. *J. Immunol.* **125**, 2137, 1980.
27. Ohsugi, Y., Nakano, T., Hata, S., Matsuno, T., Nishii, Y., and Takagaki, Y., *Chem. Pharm. Bull.* **25**, 2143, 1977.
28. Izui, S., Fernandes, G., Hara, I., McConahey, P. J., Jensen, F. C., Dixon, F. J., and Good, R. A., *J. Exp. Med.* **154**, 1116, 1981.

Received April 2, 1987; accepted with revision June 24, 1987.