CuSO₄ as an Inhibitor of B Cell Proliferation

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Abstract—In the course of investigating the mechanisms of the in vitro immunosuppressive effect of D-penicillamine in the presence of copper ion with murine spleen cells, we observed that copper ion, by itself, exhibited a strong immunosuppressive effect at a low concentration. Namely, the addition of CuSO₄ at a concentration of 0.1 to 2 μM markedly inhibited the development of SRBC-specific plaque forming cells (PFC) without causing cytotoxicity. CuSO₄, at the same concentration range, suppressed the lipopolysaccharide (LPS)-induced proliferative response, but not the response induced by concanavalin A (Con A). The suppressive effect of CuSO₄ on the antibody production was reversed by 500 U/ml of catalase, but that on the LPS-induced proliferative response was not. CuSO₄ also substantially suppressed the pokeweed mitogen-induced proliferative response. These findings suggest that CuSO₄ acts as an inhibitor of B cell proliferation and, through this effect, markedly inhibits the antibody production in murine spleen cells.

Rats with adjuvant-induced arthritis, which is known as an animal model for rheumatoid arthritis (RA), exhibit an elevated copper level in the serum and liver (1, 2). The copper concentration in the serum and synovial fluid of patients with RA is also known to be significantly elevated over normal values (3). The elevated serum and tissue copper level in RA patients is understood as a physiological response to inflammation (4), since copper is known to have potent anti-inflammatory (5–7) and antirheumatic effects (8).

Although immunological disorders have been implicated in the pathogenesis and maintenance of RA, there have been few investigations on the effect of copper on immune systems as a potential mechanism for its antirheumatic effect. Lipsky and co-workers demonstrated that D-(-)-penicillamine (D-Pc), which is well-known as a potent antirheumatic drug, exhibited its immunosuppressive effect by inhibiting the proliferative response in human peripheral T cells in the presence of copper or ceruloplasmin (9–11). Since this inhibition could be reversed by catalase, the involvement of hydrogen peroxide (H₂O₂) is suggested. Although the precise mechanism for this inhibition is not clear, Yamanaka and his colleagues showed that H₂O₂ produced inhibited the proliferative response by cleaving the DNA chain of human lymphocytes (12). During the course of investigating the immunosuppressive effect of D-Pc in the presence of CuSO₄, we observed that anti-SRBC antibody production in murine spleen cells was markedly inhibited by a low concentration of CuSO₄ even in the absence of D-Pc. In addition, CuSO₄ inhibited the proliferative responses induced by lipopolysaccharide (LPS) and pokeweed mitogen (PWM), but not that by concanavalin A (Con A). Here we report these results along with a discussion of the possible mechanisms.

Materials and Methods

Animals: Female BALB/c mice (7 weeks of age) were purchased from Charles River Japan, Inc. (Tokyo) and used during 8–14 weeks of age.

Materials: The reagents used in the present experiments were obtained from the following...
In vitro antibody production: In vitro antibody production and mitogen-induced proliferative response were determined as previously described (13). BALB/c spleen cells (8×10⁶) were cultured with SRBC (2×10⁶) in 1.5 ml of RPMI-1640 medium supplemented with 10% of FCS using 24-well, multi-dish culture plates (NUNC, Kamstrup, Denmark) at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The compounds to be tested were added at the initiation of the culture. The number of SRBC-specific plaque-forming cells (PFC) was enumerated after 4-days incubation. The viability of cultured spleen cells was determined on day 4 by the trypan blue dye-exclusion method.

In vitro mitogen-induced proliferative response: BALB/c spleen cells (2×10⁵) were cultured with LPS (20 µg/ml), Con A (5 µg/ml) or PWM (10 µg/ml) in 0.2 ml of RPMI-1640 medium containing 5% of FCS using round-bottomed microculture plates with 96 wells (NUNC) for 2 days and were pulsed with 0.5 µCi of [6-³H]thymidine ([³H]-TdR) (26 Ci/m mole, Radiochemical Center Amer- sham, England) for an additional 18 hr. Then cells were harvested on glass fiber filters [³H]-TdR incorporation was measured in a liquid scintillation counter. The compounds to be tested were added at the initiation of the culture.

Results

Effect of CuSO₄ and D-Pc on in vitro anti-SRBC PFC response: In the present study, D-Pc at 5 to 500 µM dose-dependently inhibited the anti-SRBC PFC response in BALB/c spleen cells (Fig. 1A). The inhibition by D-Pc was potentiated in the presence of 2 µM of CuSO₄. CuSO₄ at 2 µM, by itself, suppressed this response almost completely (Fig. 1B). The suppressed PFC responses of the cultures containing CuSO₄ (2 µM) alone or both CuSO₄ and D-Pc (5, 50, or 500 µM) were almost completely restored by the addition of 500 U/ml of catalase (Fig. 1C) Subsequent investigation showed that varying concentrations of CuSO₄ suppressed the anti-SRBC PFC response. CuSO₄, at 0.1 to 2 µM, dose-
dependently inhibited this response. Although the antibody production was markedly suppressed in the culture containing 2 μM CuSO₄, the viability of spleen cells in this culture was not altered (Table 1).

Effect of CuSO₄ and D-Pc on mitogen-induced proliferative response: In our study, D-Pc at 50 and 500 μM suppressed the Con A-induced proliferative response in a dose-dependent manner. This suppression was potentiated in the presence of 8 μM CuSO₄, although CuSO₄ alone did not alter the Con A response (Table 2). In the presence of 500 U/ml of catalase, however, such potentiation was not observed. In contrast to the Con A response, the LPS response was suppressed by 8 μM CuSO₄ by about 70%. This suppression was potentiated in the presence of 500 μM D-Pc. The LPS response in this culture was restored by 500 U/ml of catalase to the level of the culture containing 8 μM CuSO₄ alone, but the suppression by CuSO₄ could not be restored by catalase at all. This was quite a contrast with the preceding observation that inhibition of the anti-SRBC PFC response by 2 μM CuSO₄ was almost completely restored by 500 U/ml of catalase (Fig. 1C).

In Fig. 2, the effect of varying concentrations of CuSO₄ on either the Con A- or LPS-induced proliferative response was studied. Again the Con A response was not altered by CuSO₄ up to 8 μM, whereas the LPS response was dose-dependently suppressed by CuSO₄ at concentrations from 0.5 to 8 μM (Fig. 1).

In order to determine whether CuSO₄ also inhibits the response induced by B cell mitogens other than LPS, its effect on the PWM-induced proliferative response was studied. As shown in Fig. 3, CuSO₄ at concen-

| CuSO₄ (μM) | Anti-SRBC PFC/culture | viability (%) |
|-----------|-----------------------|--------------|
| 0         | 826±42                | 41.3±8.5     |
| 0.1       | 476±48                | ND           |
| 0.5       | 274±76                | ND           |
| 1.0       | 166±64                | 47.5±7.8     |
| 2.0       | 46±12                 | 44.5±3.8     |

BALB/c spleen cells (8×10⁶) were cultured with 2×10⁶ of SRBC in the presence or absence of CuSO₄. Anti-SRBC PFC and viability were assayed on day 4. The data represent the mean±S.D. of triplicate cultures. ND: not determined.

| Mitogen | D-Pc (μM) | CuSO₄ (μM) |
|---------|-----------|------------|
|         | none      | 0          | 8          |
|         |           | CAT        | CAT        |
| Con A   | 0         | 218.2±13.3 | 236.7±5.8  | 222.1±14.6 | 204.0±5.6 |
|         | 50        | 178.0±25.2 | 208.5±16.2 | 183.2±8.7  | 184.8±12.8 |
|         | 500       | 90.9±21.9  | 177.7±2.2  | 29.7±5.1   | 86.9±13.0  |
| LPS     | 0         | 30.5±2.3   | 31.0±1.2   | 6.6±0.8    | 7.7±1.0    |
|         | 50        | 28.5±1.3   | 27.0±1.5   | 6.8±1.0    | 6.2±0.9    |
|         | 500       | 27.6±0.9   | 35.2±3.6   | 1.7±0.4    | 5.1±0.6    |

BALB/c spleen cells (2×10⁶) were cultured with 5 μg/ml of Con A or 20 μg/ml of LPS in the presence or absence of D-Pc, CuSO₄, and catalase for 48 hr and then pulsed with 0.5 μCi of[^3]H-TdR for an additional 18 hr. The data represent the mean±S.D. of quadruplicate cultures. CAT: Catalase, 500 U/ml.
Fig. 2. Effect of various doses of CuSO$_4$ on Con A- or LPS-induced proliferative responses. BALB/c spleen cells ($2 \times 10^5$) were cultured with 5 pg/ml of Con A (○) or 20 pg/ml of LPS (○) in the presence of various doses of CuSO$_4$. The data represent the mean±S.D. of quadruplicate cultures.

Fig. 3. Effect of CuSO$_4$ on proliferative responses induced by LPS and PWM. BALB/c spleen cells ($2 \times 10^5$) were cultured with 20 μg/ml of LPS or 10 μg/ml of PWM in the presence or absence of CuSO$_4$. The data represent the mean±S.D. of quadruplicate cultures.

Concentrations from 0.5 to 8 μM also remarkably suppressed the response induced by PWM.

Discussion
In the present study, we observed that CuSO$_4$ at concentrations less than 2 μM markedly suppressed antibody production in murine spleen cells, and this inhibition was not due to its cytotoxic effect. Our results were well-consistent with those reported by
Lawrence and co-workers (14) that the SRBC-specific PFC response in murine spleen cells was suppressed by 40 to 60% by CuSO₄ at concentrations of 1 to 100 μM. However, inconsistent results were reported by Lipsky (11) that CuSO₄ at concentrations of 2 to 8 μM did not inhibit PWM-induced antibody production in human peripheral blood lymphocytes, but this response was markedly suppressed in a culture containing both D-Pc and CuSO₄. This contradictory result might result from differences in cell species, stimulatory agent, and duration of incubation with CuSO₄; that is to say, they stimulated PBMC with PWM after a 2 hr-preincubation with CuSO₄.

CuSO₄ did not inhibit the Con A response at concentrations of 0.1 to 8.0 μM (Fig. 2). In addition, we observed that the phytohemagglutinin (PHA)-induced proliferative response was not inhibited by CuSO₄ at 0.5 to 8 μM (data not shown). Recently, Smith and Lawrence reported that CuSO₄, up to 100 μM, did not inhibit IL-2 production in murine spleen cells induced by Con A (15). Taken together, the inhibitory effect of CuSO₄ on T cell proliferation seems to be insignificant.

In contrast to the responses to Con A and PHA, the proliferative responses of B cells induced by LPS and PWM were markedly suppressed by CuSO₄ (Table 2, Figs. 2 and 3). It has been reported that PWM stimulates the proliferation of T cells as well as B cells (16). In our experimental system, however, PWM intensively stimulated the proliferation of B cells, since the passage of spleen cells through a nylon-wool column decreased the PWM response by about 65%. Hence, it is conceivable that CuSO₄ inhibited the proliferative response of B cells induced by PWM. The proliferative response induced by lipid A or anti-mouse IgM (μ) antibody was also suppressed by CuSO₄ at 0.5 to 8 μM (I. Yamamoto et al., unpublished data). These findings suggest that the inhibitory effect of CuSO₄ on antibody production is at least partly due to a specific inhibition of B cell proliferation.

It was suggested that 2 μM of CuSO₄ remarkably inhibited the anti-SRBC PFC response through the production of H₂O₂ (Fig. 1C). Lipsky and Matubara demonstrated that the combination of CuSO₄ and various thiols other than D-Pc, including glutathione, also resulted in the production of H₂O₂, and in turn, in marked suppression of proliferative responses in human lymphocytes, fibroblasts, and endothelial cells (9, 17). Since the medium and FCS used in the present study contain a significant amount of glutathione (18), CuSO₄ may have suppressed the anti-SRBC PFC response through H₂O₂ production even in the absence of D-Pc.

In contrast to this, the inhibitory effect of CuSO₄ on the LPS response was not restored by 500 U/ml of catalase at all, in spite of the fact that the enhanced suppression of the Con A and LPS response observed in a culture containing D-Pc and CuSO₄ was partially restored by catalase (Table 2). These results seem to rule out the role of H₂O₂ in this suppression. The detailed mechanism through which CuSO₄ inhibits the LPS response is now under investigation.

Copper is reported to have a potent anti-rheumatic activity (8). Copper is suggested to exhibit this activity through modification of prostaglandin synthesis (19, 20), stabilization of lysosomal membrane (8), induction of superoxide dismutase (SOD) and SOD-mimetic activity (8), and induction of lysyl oxidase (21). Our results demonstrated that CuSO₄, at a concentration causing no cytotoxic effect, markedly suppressed the antibody production by mechanisms involving the inhibition of B cell proliferation. The human B cell subpopulation identified by the expression of cell surface antigen CD5 is increased in the patients with rheumatoid arthritis, and it is suggested to be responsible for most of the IgM rheumatoid factor secreted (22, 23). Copper might exhibit antirheumatic activity through specific inhibition of function(s) of B cells, including CD5⁺ B cells, although further studies are needed to evaluate this possibility.

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