Augmentation of the Antibody Response by Lipoic Acid in Mice

I. Analysis of the Mode of Action in an In Vitro Culture System

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Abstract—Lipoic acid (Lip), a naturally occurring disulfide compound, was found to augment markedly in vitro antibody responses to sheep erythrocytes (SRBC), dinitrophenyl-Ficoll and trinitrophenyl-lipopolysaccharide (TNP-LPS) as effectively as 2-mercaptoethanol (2-ME) in murine lymphocytes. The mitogenic response to LPS or concanavalin A (Con A) was augmented by Lip only slightly. 2-ME has been reported to facilitate cystine utilization by the lymphocytes, but Lip did not, indicating that the mode of action of Lip is different from that of 2-ME. Lip augmentation of anti-SRBC response was markedly abrogated when murine lymphocytes were depleted of T cells and cultured in the presence of Con A-conditioned medium containing T cell-replacing factor. The effect of Lip was also diminished in the response to TNP-LPS when the spleen cells were depleted of T cells. These observations suggest that Lip could augment the antibody response by stimulating a T cell subpopulation. This idea was confirmed by the experiment that Lip could enhance helper T cell activity which was induced by culturing murine lymphocytes with the antigen.

It has been reported that various thiol compounds including 2-mercaptoethanol (2-ME) could augment in vitro antibody responses (1–3), mitogenic responses (3, 4) and the in vitro induction of cytotoxic T cells (5, 6). We have demonstrated that one of the important roles of thiol compounds in augmenting the antibody response is to stimulate the uptake of cystine, an essential nutrient by murine lymphocytes (7, 8). Recently, we have found that the augmentation was observed with not only thiol compounds but also their oxidized forms, disulfide compounds (9, 10). It was suggested that an intramolecular disulfide compound like oxidized dithiothreitol did not stimulate B cells directly in contrast to 2-ME, but promoted these cells to differentiate into plasma cells through augmenting helper T cell activity (10).

We attempted to search disulfide compounds that can be administered into the body as an immunostimulant and found that lipoic acid (Lip), a naturally occurring disulfide, could augment the antibody response in mice both in vivo (11) and in vitro. In the present paper, the mode of action of Lip was examined by employing in vitro culture of murine lymphocytes.

Materials and Methods

Materials: Female BALB/c mice (6 weeks of age) were purchased from Shizuoka Agricultural Cooperation of Experimental Animals, and they were used during 10–15 weeks of age. Chemicals and other materials used in the present work were purchased from the following sources: DL-α-lipoic acid (Nakarai Chemicals), 2-ME and cystine dihydrochloride (Tokyo Chemical Industry), Sheep erythrocytes (SRBC) and horse erythrocytes (HRBC) (Nishinippon Sheep Farm), Keyhole limpet hemocyanin (KLH) (Calbiochemica), Ficoll 400 (Pharmacia Fine Chemicals), lipopolysaccharide of E. coli 055 B5 (LPS) (Difco), concanavalin A (Con A)
(E-Y Laboratories), monoclonal anti-Thy-1.2 (Olac 1978), (3H)-thymidine ((3H)-TdR) (RCC Amersham), Fetal calf serum (FCS) (Flow Laboratories), RPMI-1640 medium (Nissui Seiyaku). Trinitrophenyl (TN P) coupled KLH or LPS and dinitrophenyl (DNP)-Ficoll were prepared as described previously (7). Cystine-free RPMI-1640 medium was prepared by using commercial ingredients according to the composition reported by Moore et al. (12). RPMI-1640 medium was usually supplemented with penicillin G (50 units/ml) and streptomycin (50 µg/ml).

Lymphocyte culture: By using NUNC multi-dish culture plates with 24 wells, BALB/c spleen cells (6x10^6) were cultured with 2x10^3 SRBC, 10 ng/ml DNP-Ficoll or 0.5 µg/ml TNP-LPS in 1 ml of RPMI-1640 medium containing 10% FCS for 4–5 days at 37°C under 5% CO2 and 95% air. The number of plaque-forming cells (PFC) were enumerated according to the method of Jerne and Nordin (13). Unless otherwise stated, IgM PFC were enumerated. Anti-DNP and TNP PFC were assayed by using TNP-SRBC that were prepared by the method of Rittenberg and Pratt (14). In some cases, T cell-depleted spleen cells were cultured by the same procedure. T cell-depletion was carried out by treating spleen cells with anti-Thy-1.2 and guinea pig complement as described earlier (7). Cultures were performed in duplicate or triplicate. Typical data from several repeated experiments were presented as the mean or the mean±standard error. Standard error did not usually exceed 15% of the mean value.

In vitro induction and assay of helper T cell activity: BALB/c spleen cells (1x10^7) were cultured by Marbrook’s method (15) in the presence or absence of 5x10^5 HRBC for 3 days to induce helper T cell activity. Where indicated, 10^-5 M Lip was added to the culture. These cells were harvested and the viable cells were cocultured in varying numbers with 6x10^6 TNP-KLH-primed spleen cells in the presence of 5x10^5 TNP-HRBC for 5 days using a NUNC multi-dish culture plate as described above. Helper T cell activity was estimated by enumerating anti-TNP IgM PFC by using TNP-SRBC. Mice were primed with TNP-KLH by intraperitoneal injection of TNP-KLH (100 µg) adsorbed on 4 mg of alum 1–2 months prior to use.

In vitro induction and assay of suppressor T cell activity: Spleen cells (6x10^6) were cultured with 60 µg/ml KLH in 1 ml of RPMI-1640 medium containing 10% FCS for 4 days using a NUNC multi-dish culture plate. Varying numbers of viable harvested cells were cocultured with TNP-KLH-primed cells (6x10^6) in the presence of 0.1 µg/ml TNP-KLH and 10^-6 M 2-ME for 5 days. Suppressor T cell activity was estimated by the suppression of anti-TNP IgG PFC response.

Preparation of Con A-conditioned medium (Con A-sup): Con A-sup was prepared as described by Jayaraman et al. (16) with some modifications. Murine spleen cells (1x10^7) were cultured with 2.5 µg/ml Con A for 24 hr in 1 ml of RPMI-1640 medium containing 10% FCS. The collected supernatants (10 ml) were passed through Sephadex G-10 column (1x2 cm) to remove Con A and stored frozen until use. Con A-sup was added to the culture medium at 25% (v/v).

Proliferative response of murine spleen cells: BALB/c spleen cells (1x10^5) were cultured in triplicate with LPS (25 µg/ml) or Con A (2.5 µg/ml) in 0.2 ml of RPMI-1640 medium containing 5% FCS. The cells were pulsed with 0.5 µCi of (3H)-TdR for the final 18 hr of culture, and the incorporated radioactivities were measured as described previously (7).

Results

Effect of Lip on antibody responses in vitro: As shown in Fig. 1, in vitro antibody responses not only to a T cell-dependent (TD) antigen, SRBC but also to T cell-independent (TI) antigens like TNP-LPS and DNP-Ficoll were augmented markedly by Lip when it was added to the culture medium. Lip augmented the immune response optimally at 10^-5 M as effectively as 2-ME that had been shown to have the highest augmenting effects among the thiol compounds tested (3). Although the detailed data are not shown, Lip did not increase the number of nonspecific PFC in the absence of the antigen, nor did it significantly improve
the viability of the lymphocytes during the culture.

Effect of Lip on proliferative responses: Figure 2 shows the effect of Lip on the proliferative responses in murine lymphocytes. In the absence of the mitogen, Lip, itself could not induce \((^{3}H)\)-TdR uptake in contrast to 2-ME. However, \((^{3}H)\)-TdR uptake induced by Con A or LPS was stimulated by Lip. However, the stimulation index of Lip was relatively small (1.5–2.0) compared with that of 2-ME (3.0–5.0).

Effect of Lip on the dose-response profile of cystine in in vitro antibody response: The antibody response could not be elicited in cystine-free culture medium as reported previously (7). The response, however, was restored by the addition of cystine to the cystine-free medium in a dose-dependent manner as shown in Fig. 3. When 2-ME was present in the culture medium, it was observed that the dose-response curve was shifted distinctly to lower concentrations. This can be explained by our previous observations that 2-ME markedly stimulated the transport of cystine into murine lymphocytes (7–9). In contrast to 2-ME, Lip did not shift the dose-response curve. Lip-augmentation was not observed at lower concentrations of cystine (<0.1 mM). The response was augmented by Lip only when a significant response was elicited in the presence of relatively higher concentrations of cystine. This implies that Lip does not augment the antibody response by facilitating the use of cystine, an essential nutrient in contrast to 2-ME.

T cell-mediated augmentation by Lip: We have recently demonstrated that a stable intramolecular disulfide compound, oxidized dithiothreitol, could augment the in vitro
antibody response by stimulating helper T cell activity specifically (10). Thus, it was examined whether Lip acts on the lymphocytes in a similar fashion. Anti-SRBC response is T cell-dependent, and it was not elicited in T cell-depleted lymphocytes. When T cells were replaced by Con A-sup containing T cell-replacing factor, 2-ME could augment the antibody response under these conditions as effectively as in normal lymphocytes, but the augmentation by Lip was largely abrogated in T cell-depleted lymphocytes (Fig. 4).

On the other hand, 2-ME could augment the antibody response to TNP-LPS, a TI antigen, whether or not T cells were present in the lymphocyte culture. It should be noted, however, that the augmentation by Lip was markedly diminished when T cells were depleted (Fig. 5). These results suggest that Lip could augment the antibody responses to both TD and TI antigens through acting on T cells. The role of T cells in the augmentation of the antibody response to TI antigen will be discussed later.

Effect of Lip on the induction of suppressor or helper T cell activity: It was investigated whether Lip augments the antibody response by stimulating helper T cell activity or by suppressing suppressor T cell activity. Figure 6 shows the effect of Lip on the induction of suppressor T cell activity in vitro. When murine lymphocytes were precultured with a high dose of KLH, the suppressor T cell activity was induced that specifically suppressed the secondary response to TNP-KLH as described previously (17). When Lip was added to the preculture, it did not

![Fig. 4](image_url)  
**Fig. 4.** T cell-mediated augmentation of the antibody response to SRBC by Lip. A) Normal spleen cells (6×10⁶) were cultured with 2×10⁹ SRBC in the presence or absence of 10⁻⁵ M 2-ME or Lip. B) T cell-depleted spleen cells (3×10⁶) were cultured with 2×10⁹ SRBC in the presence of Con A-sup (25% v/v). Where indicated, 10⁻⁵ M 2-ME or Lip was added to the culture. In one experiment indicated in the figure, Con A-sup was omitted from the culture.

![Fig. 5](image_url)  
**Fig. 5.** T cell-mediated augmentation of the antibody response to TNP-LPS by Lip. Normal (Panel A) or T cell-depleted (Panel B) spleen cells were cultured with 0.5 μg/ml TNP-LPS in the presence or absence of 10⁻⁵ M 2-ME or Lip.

![Fig. 6](image_url)  
**Fig. 6.** Effect of Lip on the induction of suppressor T cell activity in vitro. Suppressor T cell activity was induced by culturing 6×10⁹ spleen cells with 60 μg/ml KLH for 4 days in the presence or absence of 10⁻⁶ M Lip. KLH-specific suppressor T cell activity was estimated by the degree of suppression of the secondary antibody response to TNP-KLH (see Materials and Methods).
stimulate or inhibit the induction of suppressor T cell activity as illustrated in Fig. 6.

On the other hand, murine lymphocytes were precultured with HRBC, and then cocultured with TNP-KLH-primed cells in the presence of TNP-HRBC as the antigen (Fig. 7). In this type of experiment, one can estimate the induction of HRBC-specific helper T cell activity. The highest helper activity was induced when the cells were precultured with both HRBC and Lip. In the absence of HRBC, Lip alone could not significantly stimulate the helper activity, thus indicating that Lip augmented the antigen-stimulated helper T cells specifically.

Discussion

The present results demonstrated that Lip augmented the antibody response in murine lymphocytes by stimulating helper T cell activity. These resemble the augmenting effect of oxidized dithiothreitol, another intramolecular disulfide that can enhance antibody responses to both TD and TI antigens only in the presence of T cells (10). On the other hand, 2-ME appears to stimulate B cells directly because T cell-depletion did not affect the augmentation by 2-ME of the responses to SRBC and TNP-LPS in contrast to Lip or oxidized dithiothreitol (10). Interestingly, the augmentation by Lip was found to be dependent on T cells even in the antibody response to TNP-LPS that is essentially a TI antigen. Recently, several authors have reported that even the response to TI-antigen was modulated by T cells, although a basal response could be elicited in the absence of T cells (18, 19). Although the helper T cells that can augment the responses to TI antigens remain to be characterized, we have observed that the helper T cell was stimulated optimally in the presence of both LPS and oxidized dithiothreitol and could augment the antibody response to TNP-LPS (10).

The helper T cells that were stimulated by Lip in the presence of HRBC was found to be antigen-specific because they could augment the response to TNP-HRBC, but not the response to an irrelevant antigen like TNP-ovalbumin (data not shown).

It has been known that Lip plays a key role in biological systems as the coenzyme of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (20). Lip is interconvertible with its reduced form, dihydrolipoic acid during the catalysis. Therefore, Lip might act on the lymphocytes after being converted to the reduced form in the cells. In our preliminary experiments, dihydrolipoic acid showed a similar dose-response to Lip in the augmentation of the antibody response to SRBC in vitro. At the present stage, we can not conclude which form is really responsible for the augmenting effect.

It has been well-known that various thiol and disulfide compounds could stimulate the growth of lymphoma cell lines (21) and in vitro antibody responses (1-3, 7-9). It seems worth while to search among these sulfur-containing compounds for an effective immunostimulant that is excellent for therapeutic use. Lip is a naturally occurring disulfide compound, and it has been reported to be effective for the therapy of liver damages and heavy metal poisoning (22). The immunostimulating activities of Lip reported here is a novel pharmacological effect of this compound. We then investigated the effect of Lip in vivo, and we report in the accompanying paper that Lip was effective in restoring the suppressed
immune responses when administered to mice (11).

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