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Mechanism of Adjuvant Activity of Dental Plaque: In Vitro Activation of Residual Helper T-Cell Precursors in T-Cell-Deficient Murine Spleen Cell Cultures

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The immunoenhancing activity of a water-soluble extract of dental plaque (DP), which contains a mixture of microbial antigens, has been investigated. DP was tested for its capacity to augment the in vitro antibody-forming cell (AFC) response to sheep erythrocyte by adherent spleen cells from thymectomized, lethally irradiated, and bone marrow-transplanted (TxB) mice. Although DP was found to induce a small polyclonal AFC response, most of the increase in AFC induced by DP was antigen dependent. The latter enhancing effect is an indicator of the adjuvanticity of DP. This adjuvant activity of DP was T-cell-dependent, since removal of the residual prethymic and/or thymic-derived lymphocytes (T-cells) by anti-T-cell serum (anti-θ) and guinea pig complement abrogated the capacity of DP to augment the in vitro AFC response. This view was further supported by the synergistic restorative effect obtained by culturing anti-θ-treated adherent spleen cells with both DP and a population of unactivated T-cells that by themselves were unable to significantly enhance AFC responsiveness. Moreover, DP was found to be mitogenic for thymocytes. The cumulative results suggest that the adjuvant activity of DP is dependent on both the T- and B-cell-activating components present in DP.

Dental plaque (DP) consists of an aggregation of a large number of oral bacteria which attach tenaciously to teeth and accumulate predominantly at the junction between the tooth and the gingiva. Accumulation of DP results in the development of gingivitis, and subsequent removal of DP results in reversal of the gingivitis (11). It is therefore likely that DP contributes to gingival inflammation by serving as a direct toxic as well as an antigenic irritant of the adjacent tissue and/or as a nonspecific adjuvant to the local lymphoid elements. The adjuvant properties of DP have, in fact, been detected by Lehner et al. (10), who demonstrated increased blastogenic responses to phytohemagglutinin and unrelated antigens such as purified protein derivative during experimental gingivitis in humans. Further support for the concept of an adjuvant effect of DP was provided by Reed et al. (19), who observed that DP and phytohemagglutinin had a synergistic effect on the blastogenic response of human and monkey lymphoid cells.

The gingiva in chronic periodontal disease is characterized histologically by a predominantly plasma cell infiltrate (20), which indicates local antibody synthesis. It is therefore possible that DP might also be serving as an adjuvant for this immune response. We have therefore examined the ability of a soluble extract of DP to augment an antibody response to an unrelated thymic-dependent antigen in an animal model. This has been done by utilizing a modification of the in vitro immunization procedure of mouse spleen cells, which allows separation of the various lymphoid components involved in the antibody response. The results suggest that the adjuvant effect of DP is, in part, dependent on its capacity to activate precursors of helper T-cells, which, in turn, are contributing to the activation of the antibody-forming cell (AFC) precursors.

MATERIALS AND METHODS

Animals. Male and female BC3F$_1$/Cum (C57BL × C3H/AnF) mice were obtained from Cumberland View Farms (Clinton, Tenn.). C3H/HeJ mice were obtained from Jackson Laboratories (Bar Harbor, Maine). The mice were maintained as previously described (4). T-cell-deficient (TxB) mice were prepared by thymectomizing adult animals, followed by lethal irradiation (800 R) and injection of syngeneic bone

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marrow cells (5×10^6), as previously decribed (5). The spleen cells from the TxB mice were used 8 to 12 weeks after bone marrow reconstitution.

Antigen. Sheep erythrocytes (SRC) were obtained from the Colorado Serum Co. (Denver, Colo.). The SRC were prepared for use in the tissue culture immunization procedure as previously described (4).

Adjuvant. DP was collected by scaling the teeth of dental patients. The DP was pooled, washed in phosphate-buffered saline, and sonically treated for 20 min at 4°C. The sonic extract was centrifuged at $10{,}000 \times g$ for 30 min at 4°C, lyophilized, and subsequently adjusted to 100 μg of DP protein per ml, as determined by the Lowry protein assay, using bovine serum albumin as the standard (13). The DP solution was sterilized by filtration through a 0.45-μm filter (Nalge Corp., Rochester, N.Y.) and stored at −70°C until used.

Anti-T-cell serum. AKR/J anti-C3H/HeJ anti-theta (θ) serum was prepared as previously described (4).

Preparation of T-cell-deficient mouse spleen cell cultures. Adherent cell monolayers were prepared from the spleens of the TxB mice according to the method of Mosier (17). It is known that adherent spleen cell monolayers prepared by this procedure contain macrophages (17) and also AFC precursors (12). The failure of this cell preparation to respond to the T-cell-dependent antigen, SRC, is therefore due to the absence of significant numbers of T-cells. Further depletion of the residual T-cells was accomplished by treating the adherent spleen cells with anti-θ and guinea pig complement (Texas Biologicals, Ft. Worth, Tex.). The adherent spleen cell monolayers were exposed to 1 ml of a 1/10 dilution of the anti-θ serum for 30 min at 37°C. After this incubation, the cells were washed and treated with 1 ml of a 1/10 dilution of guinea pig complement for 30 min at 37°C. The monolayers were then washed repeatedly with tissue culture medium containing 10% fetal calf serum before the addition of antigen, as described below.

Preparation of activated and unactivated T-cells. SRC-activated T-cells (ATC_{SRC}) were prepared by passaging thymocytes with SRC through lethally irradiated syngeneic recipients, as previously described (15). Unactivated T-cells (UTC) were similarly prepared except that the SRC were not included. The ATC_{SRC} and UTC were further purified by elution from a nylon wool column as previously described (4).

In vitro induction of the antibody response to SRC. The TxB adherent spleen cell monolayers with or without ATC_{SRC}, UTC, or DP were immunized with 5×10^6 SRC and incubated for 4 days, using the culture conditions described by Mishell and Dutton (16).

Hemolytic AFC assay. On day 4 of incubation, the immunoglobulin M antibody response of the bone marrow-derived lymphocytes (B-cells) from the adherent spleen cell monolayers were assessed by enumerating the AFC by a modification (18) of the hemolytic plaque-forming cell assay of Jerne and Nordin (8). To simplify the presentation of the data and to facilitate direct comparison of data from different experiments, the number of AFC present in adherent spleen cell cultures containing ATC_{SRC} and SRC has been normalized to 1,000 AFC per culture. All the other values within the experiment are then expressed as relative AFC per culture ± standard error. The mean absolute AFC per culture response of the positive control culture containing spleen cell monolayers, ATC_{SRC}, and SRC is also presented in each figure.

In vitro thymocyte mitogenesis. Thymocyte mitogenesis was assayed according to methods previously described (9). Thymuses were aseptically removed from 4- to 6-week-old C3H/HeJ mice and gently pressed through a 60-mesh, stainless-steel, wire mesh screen into RPMI 1640 media supplemented with a final concentration of 2.5×10^{-5} M 2-mercaptoethanol, 12 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid), penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and 5% fetal calf serum. A total of 1.5×10^6 thymocytes in 0.1 ml was added to each well of flat-bottom microculture plates (no. 3040, Falcon Plastics, Oxnard, Calif.). Various concentrations of DP were then added in 0.1 ml of quadruplicate wells. On day 3, the cultures were pulsed for the final 4 h of incubation with 0.5 μCi of tritiated thymidine (specific activity, 2.0 Ci/mmol; Amersham, Buckinghamshire, England). Cells were collected on glass fiber filters (934 AH, Reeve Angel, Clifton, N.J.) with an automatic harvester. The filters were dried, added to 3 ml of Hydromix scintillation fluid (Yorktown Research, Hackensack, N.J.), and counted in a Packard scintillation counter.

RESULTS

The adjuvant activity of DP has been investigated by examining its capacity to augment the plaque-forming cell response of adherent mouse spleen cells from TxB mice. It has previously been shown that normal adherent spleen cells (12) as well as unfractionated TxB mouse spleen cells (5) are incapable of significant antibody responses to the thymic-dependent antigen SRC because of the paucity of T-cells in these splenic populations. The use of adherent spleen cells from TxB mice therefore assures the T-cell deficiency of these cultures. Groups A through D of Fig. 1 clearly demonstrate the ability of 10^6 ATC_{SRC} to restore the antigen-dependent AFC response of adherent spleen cell monolayers from TxB mice. Group E shows that DP is also capable of augmenting the AFC response of immunized adherent spleen cell monolayers. DP also induced a minimal but significant AFC response in the absence of the specific antigen SRC (group F), which suggested that DP contained a component(s) capable of directly activating B-cells.

Although the TxB mouse adherent spleen cell monolayers are T-cell deficient, it was still possible that DP might be directly or indirectly activating the small number of residual T-cells and/or T-cell precursors remaining in the adherent spleen cell monolayers and that this T-

cell activation was crucial to the subsequent induction of the antibody response to SRC.

We therefore tested the adjuvant effect of DP on the in vitro AFC response after treatment of the adherent spleen cell monolayers with anti-θ and guinea pig complement to remove the residual T-cells. All of the data in this experiment (Fig. 2) were from cultures that contained SRC. Groups A through D show that ATC_{SRC} enhanced the AFC response of both the untreated and anti-θ-treated adherent cells. Although in this experiment the B-cells in the untreated adherent cell monolayer yielded almost twice the number of AFC as the treated cell monolayer, this quantitative relationship has not been an entirely consistent finding. In other experiments, the response of the anti-θ-treated cells in the presence of ATC_{SRC} was greater than that of the untreated cells. The point that should be emphasized is that ATC_{SRC} are able to significantly enhance the response to SRC either in the presence or absence of residual T-cells. In contrast to the results obtained with ATC_{SRC}, groups E and F show that removal of the residual T-cells caused a greater than 95% reduction in the number of AFC produced in the presence of DP. Thus, the enhancement of the AFC response, after exposure to DP, is clearly dependent on the presence of a small number of residual splenic T-cells, which by themselves are incapable of significantly enhancing the AFC response.

To further test the T-cell dependence of the adjuvant effect of DP, anti-θ-treated adherent spleen cell monolayers were immunized with SRC in the presence of UTC, DP, or both (Fig. 3). As seen previously, the AFC response of the B-cells was restored by the addition of ATC_{SRC} (groups A and B). The response of the B-cells in the presence of DP, although significant, was clearly reduced (group C). Group D shows that the B-cells completely failed to respond, even in the presence of UTC. A significant synergistic effect was, however, obtained by culturing the B-cells with both UTC and DP (group E).

The results suggested that DP was contributing to the activation of the residual splenic helper T-cells that were present in the TxB adherent spleen cell monolayers. Recent results from our laboratory have shown that a purified lymphokine, which augments the AFC response of adherent spleen cell monolayers, is inseparable from a factor that induces thymocyte proliferation (9). These results have suggested that the ability of a biological agent or factor to induce thymocyte proliferation may, in part, reflect its ability to activate helper T-cells. We therefore tested the possibility that DP was

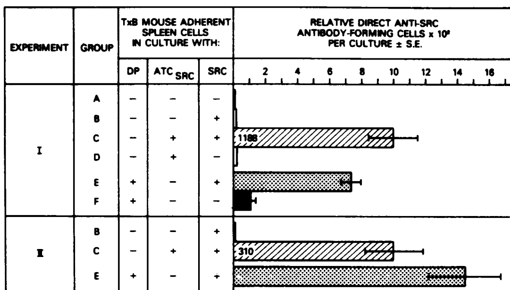

FIG. 1. *Restoration of the in vitro AFC response of adherent spleen cells from T-cell-deficient mice by dental plaque. Adherent spleen cell monolayers from TxB mice were prepared and incubated with various combinations of DP (10 μg/ml), ATC_{SRC} (0.5 × 10^6), and SRC. All of the cultures were assayed for anti-SRC immunoglobulin M-secreting AFC after 4 days of incubation. The values in the positive control bars are the absolute number of AFC generated per culture. S.E., Standard error.*

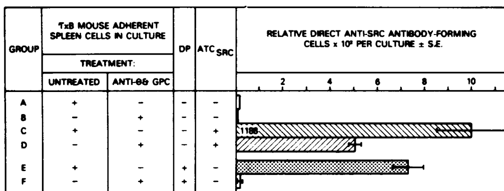

FIG. 2. *Effect of anti-θ and guinea pig complement (GPC) treatment of T-cell-deficient spleen cells on their responsiveness to DP. Adherent spleen cells from TxB mice were either treated with anti-θ and GPC or left untreated. The remaining cells were then incubated in the presence of SRC and either DP (10 μg/ml), ATC_{SRC} (0.5 × 10^6), or control medium. All of the cultures were assayed for anti-SRC immunoglobulin M-secreting AFC after 4 days of incubation. The value in the positive control bar is the absolute number of AFC generated per culture.*

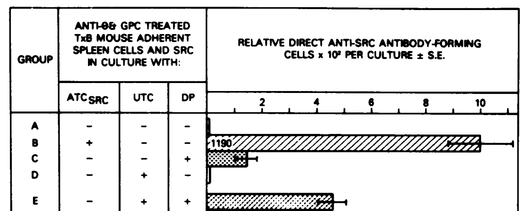

FIG. 3. *Synergistic helper effects of DP and unactivated T-cells on the AFC responsiveness of mouse B-cells. Adherent spleen cells from TxB mice were treated with anti-θ and GPC and then co-cultivated with SRC and either ATC_{SRC}, UTC, DP, or UTC and DP together. All of the cultures were assayed for anti-SRC immunoglobulin M-secreting AFC after 4 days of incubation. The value in the positive control bar is the absolute number of AFC generated per culture.*

either (i) capable of inducing the formation by the adherent splenic macrophages of lymphocyte activating factor, which has been shown to induce thymocyte proliferation (6), or (ii) capable of directly inducing thymocyte proliferation. Although we were unable to demonstrate enhanced lymphocyte activating factor production by DP-stimulated splenic macrophages, the data of Fig. 4 clearly show that some component of DP exhibits a dose-dependent mitogenic effect on mouse thymocytes.

DISCUSSION

It has been suggested that soluble products from dental plaque may contribute to the exacerbation of the chronic inflammatory lesion associated with periodontal disease (11). Mackler et al. (14) have shown that DP can activate human T- as well as B-cells. The high frequency of plasma cells in the lymphocyte infiltrate within the lesion (20) suggests that antibody synthesis represents an important component of the inflammatory process and thus raised the question as to the adjuvanticity of DP.

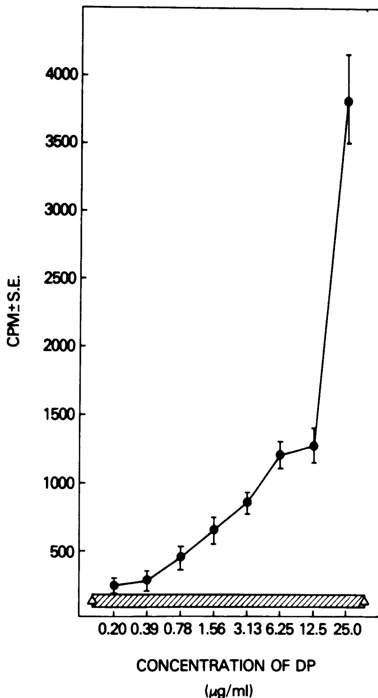

FIG. 4. *DP-induced mitogenesis of murine thymocytes. A total of 1.5 × 10^6 murine thymocytes were cultured in 0.2 ml of tissue culture medium containing increasing concentrations of DP. The cultures were assayed for incorporation of tritiated thymidine on day 3 of incubation after a 3-h pulse with tritiated thymidine. The horizontal shaded bar at the bottom of the figure represents the response of those cultures that were not exposed to DP.*

The results of this investigation have clearly shown that DP is a potent adjuvant during the in vitro antibody response to an unrelated antigen and that DP is capable of partially replacing the requirement for helper T-cells (Fig. 1). However, the introduction of DP into T-cell-deficient cultures did not completely override the requirement for helper T-cells (Fig. 2 and 3), which clearly suggested that at least one of the components of DP was involved in the activation of these T-cells. The presence of a T-cell-activating component(s) in DP was further supported by the data that showed that DP was capable of inducing mitogenesis of thymocytes in vitro (Fig. 4). The presence of T-cell-activating components in DP is consistent with the observations of Lehner et al. (10) and Reed et al. (19) that DP has adjuvant effects. Furthermore, our results are similar to those of Hamaoka and Katz (7), who showed that a variety of adjuvants exerted their adjuvant activity by activating the small number of unimmunized carrier-reactive T-cells during an adoptive antihapten antibody response. However, our results apparently differ from those of Baker et al. (1), who showed that a water-soluble supernatant fraction of BCG could stimulate B lymphocytes to form anti-SRC antibody when T lymphocytes were depleted or absent, and from the results of Campbell et al. (3), who have similarly demonstrated that a cell wall-rich fraction of *Listeria monocytogenes* stimulated B-cells, in the absence of T-cells, to respond to SRC. However, these conclusions that the site of action of an adjuvant is directly on the B-cells and that activation of T-cells is not required are dependent on having absolutely pure populations of B-cells, which are virtually impossible to obtain.

Conversely, our finding that DP contains a component capable of inducing a small but significant amount of polyclonal activation (Fig. 1) suggests that DP may also be capable of directly activating B-cells, which is consistent with the presence of small amounts of endotoxin in DP (70 ng/ml by limulus assay). The suggestion might therefore be made that the total adjuvant activity of DP, and hence the maximal response of the TxB adherent spleen cells, might be dependent upon separate T-cell-activating and B-cell-activating components in DP. Studies characterizing the different bacterial components in DP are currently in progress.

The preparation of DP used in these experiments was not subjected to an extensive bacteriological analysis. However, a recent study showed that *Actinomyces viscosus* was present in the DP of 10 out of 10 individuals studied (2).

It, therefore, seems virtually certain that our preparation, which was a pool of DP from approximately 100 individuals, would contain *A. viscosus*. Recent experiments have shown that a cell wall extract of *A. viscosus* induces significant antigen-dependent antibody formation in vitro by spleen cells from congenitally athymic nude mice (P. Chen, J. J. Farrar, and G. J. Genco, manuscript in preparation). This suggests that a component of the cell wall of this organism may account for the adjuvant effects of DP.

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LITERATURE CITED

1. **Baker, L. A., T. Sharpton, P. Munden, and P. A. Campbell.** 1976. Adjuvant and mitogenic properties of a supernatant fraction of sonically treated *Mycobacterium bovis* (BCG). Infect. Immun. **14**:83-87.
2. **Bowden, G. H., J. M. Hardie, and G. L. Slack.** 1975. Microbial variation in approximal dental plaque. Caries Res. **9**:253-277.
3. **Campbell, P. A., C. Schaffler, and G. E. Rodriguez.** 1976. Listeria cell wall fraction: a B cell adjuvant. J. Immunol. **116**:590-594.
4. **Farrar, J. J., and J. Fuller-Bonar.** 1976. The xenogeneic effect. II. Requirement for unactivated murine T cells during restoration of immune responsiveness with xenogeneic reconstitution factor. J. Immunol. **117**:274-282.
5. **Farrar, J. J., B. E. Loughman, and A. A. Nordin.** 1974. Lymphopoietic potential of bone marrow cells from aged mice. Comparison of the cellular constituents of bone marrow from young and aged mice. J. Immunol. **112**:1244-1249.
6. **Gery, I., and B. Waksman.** 1972. Potentiation of the lymphocyte response to mitogens. II. The cellular source of potentiating mediators. J. Exp. Med. **136**:143-155.
7. **Hamaoka, T., and D. H. Katz.** 1973. Cellular site of action of various adjuvants in antibody responses to hapten-carrier conjugates. J. Immunol. **111**:1554-1563.
8. **Jerne, N. K., and A. A. Nordin.** 1963. Plaque formation in agar by single antibody-producing cells. Science **140**:405.
9. **Koopman, W. J., J. J. Farrar, J. J. Oppenheim, J. Fuller-Bonar, and S. Dougherty.** 1977. Association of a low molecular weight helper factor(s) with thymocyte proliferative activity. J. Immunol. **119**:55-60.
10. **Lehner, T., J. M. A. Wilton, S. J. Challacombe, and L. Ivanyi.** 1974. Sequential cell mediated immune responses in experimental gingivitis in man. Clin. Exp. Immunol. **16**:481-492.
11. **Loe, H., E. Theilade, and S. B. Jensen.** 1965. Experimental gingivitis in man. J. Periodontol. **36**:117-187.
12. **Loughman, B. E., J. J. Farrar, and A. A. Nordin.** 1974. Presence of antibody-forming cell precursors in the mouse adherent spleen cell population. J. Immunol. **112**:430-432.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
14. **Mackler, B. F., L. C. Altman, S. Wahl, D. L. Rosenstreich, J. J. Oppenheim, and S. E. Mergenhagen.** 1974. Blastogenesis and lymphokine synthesis by T and B lymphocytes from patients with periodontal disease. Infect. Immun. **10**:844-850.
15. **Miller, J. F. A. P., and G. G. Mitchell.** 1968. Cell to cell interaction in the immune response. I. Hemolysin forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. J. Exp. Med. **128**:801-820.
16. **Mishell, R. I., and R. W. Dutton.** 1967. Immunization of dissociated spleen cell cultures from normal mice. J. Exp. Med. **126**:423-442.
17. **Mosier, D. E.** 1967. A requirement for two cell types for antibody formation *in vitro*. Science **158**:1573-1575.
18. **Plotz, P. H., N. Talal, and R. Asofsky.** 1968. Assignment of direct and facilitated hemolytic plaques in mice to immunoglobulin classes. J. Immunol. **100**:744-749.
19. **Reed, M. J., M. E. Neiders, and R. J. Genco.** 1976. Synergistic effects on DNA synthesis in peripheral blood lymphocytes of mitogens and dental plaque sonicates in man and Macaque monkeys. Arch. Oral Biol. **21**:441-445.
20. **Schlossberg, A., and P. A. Ferrigino.** 1971. A histological study of the incidence of plasma cells and lymphocytes in human gingival tissue. J. Oral Med. **26**:99-105.