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Modulation of Delayed-Type Hypersensitivity and Cellular Immunity to Microbial Vaccines: Effects of Cyclophosphamide on the Immune Response to Tularemia Vaccine

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Treatment of guinea pigs with cyclophosphamide before immunization with killed tularemia vaccine in Freund incomplete adjuvant produced a prolongation and intensification of delayed-type hypersensitivity and in vitro lymphocyte transformation reactions to tularemia antigen. Such reactions resemble those ordinarily associated with the administration of live tularemia vaccine, killed vaccine in Freund complete adjuvant, or recovery from natural infection. The immunopotentiation lasted longer than that seen previously in other antigenic systems with this drug and was dependent on the dose of vaccine used. More intense delayed skin reactivity could be transferred into normal controls by cells from immunized donors pretreated with cyclophosphamide than by cells from immunized donors that were not pretreated.

The bacterial infection tularemia has been widely studied in humans and in experimental animals as a model system for assessing the relationship between cell-mediated immunity and resistance to infection. Early studies demonstrated the appearance of delayed-type hypersensitivity (DTH) reactions in convalescent patients (10). In later studies a killed vaccine was shown to be ineffective in both inducing protection and DTH (8, 11, 16, 28, 29). A live vaccine was then developed that gave protection as well as intense DTH reactivity (8, 28, 29). The cellular immune nature of this resistance was established by passive transfer studies in rats and mice (7, 23). Recent experience with DTH testing in humans has been extensively reviewed (4).

Regulation of the cellular arm of the immune system by specific or nonspecific means has recently received attention. One pharmacological agent, cyclophosphamide (CY), widely used as a cytotoxic agent in cancer chemotherapy, has been shown to induce an increase in certain forms of DTH in guinea pigs and mice, when administered as a single large dose before immunization (22, 24, 33, 34). This action appears to be related to the selective depletion of lymphocytes having some of the characteristics of B cells (18, 27). In the present investigation we have studied the effect of CY pretreatment on the response of guinea pigs to a killed tularemia vaccine. It was found (i) that the enhancing effect of CY on DTH and cell-mediated immunity may be seen in a bacterial system, (ii) that the potentiation by CY of cell-mediated immunity to tularemia is longer lived than that seen with protein antigens, and (iii) that the increase in intensity and persistence of DTH is dependent on the dose of antigen administered. We have also demonstrated that peritoneal exudate cells (PEC) from CY-pretreated donors transfer more intense DTH reactivity than cells from donors not pretreated.

MATERIALS AND METHODS

Animals. Outbred Hartley strain guinea pigs of either sex, weighing 350 to 500 g, were used. The animals were purchased from A. Tuck & Sons Ltd., Rayleigh, Essex, U.K. They were fed on a pelleted diet RGP (E. Dixon & Son, Ware, Herts., U.K.) liberally supplemented with cabbage and hay.

Tularemia vaccine. A lyophilized tularemia Foshay-type vaccine (11) was prepared in 1975 by Henry Eigelsbach at the U. S. Army Medical Research Institute of Infectious Diseases. It consists of a phenol-Merthiolate-treated suspension of *Francisella tularensis*, strain SCHU S4, at a concentration of 7.5 × 10^9/ml and 98 μg of bacterial nitrogen per ml.

Preparation of skin test and lymphocyte transformation antigen. For skin tests and addition to lymphocyte cultures, the vaccine was diluted 1:10 in sterile saline for injection USP and dialyzed against 100 volumes of sterile saline to remove preservatives. It was compared with a sample lot of the original Foshay vaccine by LT tests in immune humans and skin tests in immune guinea pigs and shown to be of equivalent potency.

Treatment with CY. CY (Endoxana) was kindly

donated by WB Pharmaceuticals Ltd., Bracknell, Berks., U.K. It was dissolved in 0.15 M NaCl and injected in a dose of 250 mg/kg intraperitoneally 3 days before immunization.

Immunizations. The vaccine was used either undiluted or diluted 1:10. These suspensions were combined with an equal volume of saline and then emulsified in an equal volume of either Freund incomplete adjuvant (FIA; Difco) or Freund complete adjuvant (FCA [Difco]; containing *Mycobacterium butyricum*). Animals received a total of 0.4 ml of the appropriate material as four 0.1-ml injections into the footpads. These immunizing doses correspond to approximately 10 and 1 μg of bacterial nitrogen, respectively.

Skin tests. Animals were skin tested by intradermal injection of 0.1 ml containing 1 μg of bacterial nitrogen of tularemia antigen into the shaved flank. Reactions were assessed by measuring the diameter and intensity of erythema and the increase in skin thickness, using skin callipers (Schnelltäster A02T, Kröplin). Skin reactions were read at 4, 24, 48, 72, and 96 h. Unimmunized animals were similarly skin tested in parallel with the experimental animals. A slight increase in skin thickness in the controls was seen only at 4 h, and this was subsequently subtracted from the readings in the experimental animals. The results are expressed as specific increase in skin thickness, which represents the reading (0.1 mm) at the skin test site minus the average thickness of normal skin on both sides of the site.

Cell transfers. Spleen cells and PEC were obtained from immunized donors 8 days after sensitization by the procedures described previously (19). Viable PEC (2×10^8 by trypan blue exclusion) or spleen cells (3×10^8) were injected intravenously into normal recipients; skin tests were performed 1 h after injection of cells.

LT test. A whole-blood microculture technique was employed as previously used in guinea pigs (R. H. Kenyon and M. S. Ascher, Fed. Proc. 35:337, 1976). In brief, 1 ml of heart blood was obtained from unanesthetized animals and 30 U of preservative-free heparin per ml was added (Schwarz/Mann, Orangeburg, N.Y.). The blood was then immediately diluted with 9 volumes of RPMI 1640 medium (GIBCO, Glasgow, Scotland) containing 100 U of penicillin per ml and 100 μg of streptomycin per ml. Cell suspension (200 μl) was then added to wells of U-bottom microtiter plates (Sterilin). Antigen was added in a 25-μl volume to a final concentration of 0.1 μg of bacterial nitrogen per ml. After 4 days at 37°C in 5% CO_2, 0.02 μCi of [^{14}C]thymidine (Radiochemical Centre, Amersham, Bucks., U.K.) was added, and 18 h later the cells were harvested on a multiple automated sample harvester (MASH II, Microbiological Associates, Rockville, Md.). Radioactivity was quantitated with a Packard beta-scintillation counter (model 2405). Counts are expressed as the geometric mean counts per minute of quadruplicate cultures. The average standard deviation of counts in this system was 20% of the mean, and differences of twofold are significant ($P < 0.05$).

Experimental design and presentation of data. Groups of at least five animals were used in each experiment. The data are plotted as the arithmetic mean plus and minus the standard error of the mean.

RESULTS

Figure 1A shows the skin reactivity to tularemia (Tul) antigen 1 week after guinea pigs were immunized with 10 μg of bacterial nitrogen in FIA (FIA-Tul) or in FCA (FCA-Tul) compared with that of animals pretreated with CY and immunized with FIA-Tul (CY-FIA-Tul). The skin reactivity in the CY-FIA-Tul animals at all times was much greater than that seen in FIA-Tul animals and approximates that seen in FCA-Tul animals. By week 2 after immunization (Fig. 1B), an increase in 24-h reactivity in the FIA-Tul animals obscured any CY effect. However, the 48- and 72-h skin reactions of the CY-FIA-Tul animals differed from those in the untreated animals. This difference had disappeared by week 6 after immunization. At the lower immunizing dose (1 μg of bacterial nitrogen) of antigen, the increase in skin reactivity due to CY pretreatment persisted through week 2 after immunization (Fig. 2A and B). In other experiments we repeatedly skin tested the FCA-Tul- and FIA-Tul-immunized guinea pigs for 6 weeks, and at no time was any 4-h (Arthus) reactivity detected.

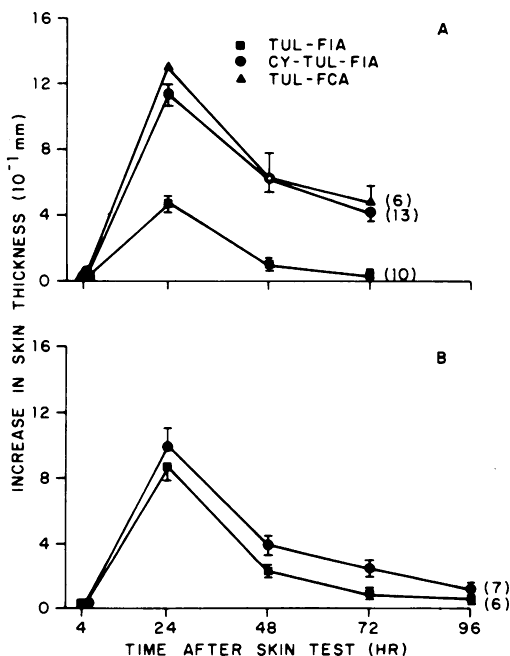

FIG. 1. *Skin test reactivity, as measured by increase in skin thickness 1 (A) or 2 (B) weeks after immunization with 10 µg of tularemia antigen. Groups of animals received FIA-Tul (■), treatment with CY 3 days before immunization with FIA-Tul (●), or immunization with FCA-Tul (▲). Numbers of guinea pigs are shown in parentheses.*

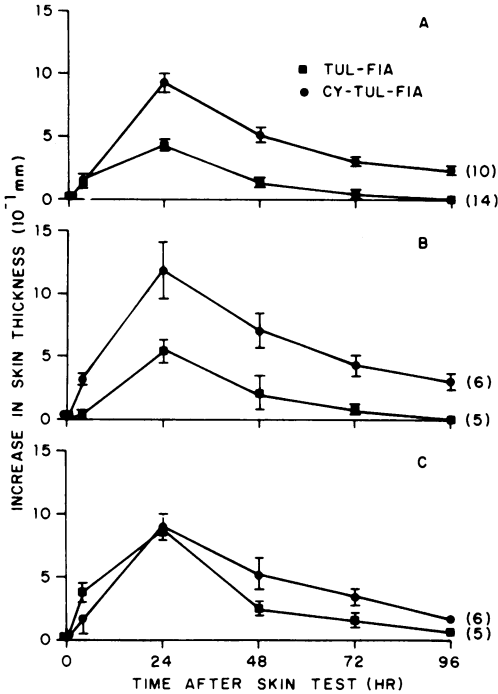

FIG. 2. *Skin test reactivity, as measured by increase in skin thickness 1 (A), 2 (B), or 3 (C) weeks after immunization with 1 μg of FIA-Tul either with (●) or without (■) CY pretreatment.*

Using this model of CY immunopotentiation to tularemia, we prepared donors for cellular transfer experiments along the lines of the classical Chase model (6). Spleen cells or PEC taken from guinea pigs 8 days after immunization with 10 μg of bacterial nitrogen in FIA (one group of donors had received CY) were transferred into normal recipient guinea pigs 1 h before skin testing. Skin test results (Fig. 3) showed only minimal activity in the recipients of either spleen cells or PEC from immunized donors not pretreated with CY or spleen cells from immunized CY-pretreated donors. A significantly higher degree of reactivity, as measured by skin thickness, was seen in the recipients of PEC from CY-pretreated donors. An increase in skin reactivity of recipients of spleen cells from CY-pretreated donors over the recipients of spleen cells from nontreated donors was even more apparent when erythema was considered as the end point, but this difference was only minimally reflected in the thickness data. A dissociation between erythema and induration has been seen previously as a feature of CY pretreatment (30).

Figure 4 illustrates the LT responses to tularemia antigen at varying times after immunization of the animals depicted in Fig. 1. The background (unstimulated) culture response was elevated in the CY-FIA-Tul group on day 11 but had returned to the level of that found in the FIA-Tul animals by day 28. Despite the transient elevated background, the response to the tularemia antigen was higher in the CY-pretreated animals at all times tested.

DISCUSSION

Early experiments on the effects of CY on the cellular immune response used contact sensitizers and purified proteins such as ovalbumin as test antigens (33, 34). Attempts to show immunopotentiation by CY to a microbial antigen, tuberculin, in BCG-immunized guinea pigs were unsuccessful (34). Other workers have used CY in model infections in varying doses and schedules and animal species. In general, CY given at the time of infection or later interferes with resistance. Such effects have been seen with a number of viruses, such as influenza (14), Sendai (2), West Nile encephalitis (5), Semliki Forest disease (3), rabies (17), and Venezuelan equine

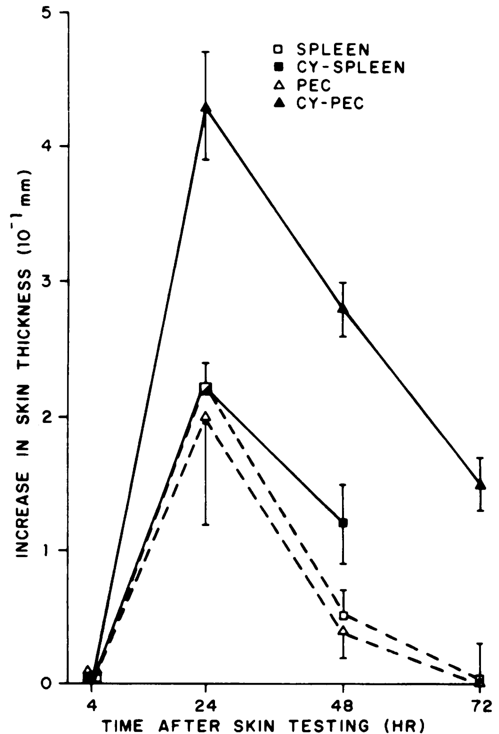

FIG. 3. *Transfer of PEC and spleen cells. Donor cells were taken 8 days after immunization with 10 μg of FIA-Tul with or without CY. Recipient animals received FIA-Tul PEC (△), CY-FIA-Tul PEC (▲), FIA-Tul spleen cells (□), or CY-FIA-Tul spleen cells (■).*

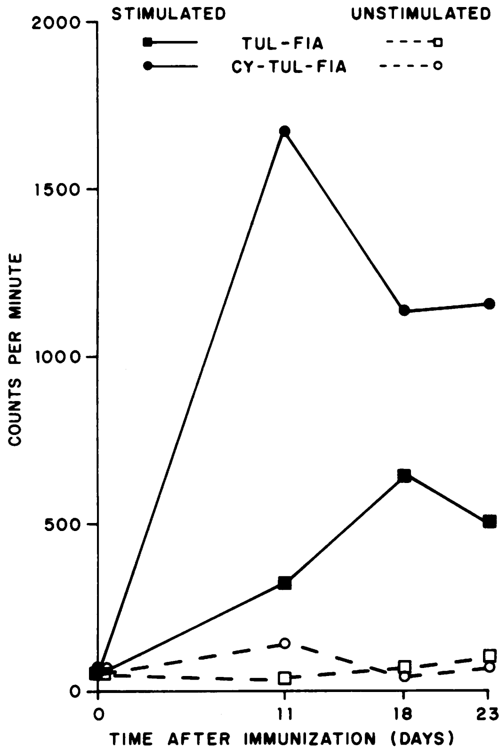

FIG. 4. *LT responses of animals immunized with 10 μg of FIA-Tul with or without CY pretreatment. The unstimulated (no antigen added) and stimulated cultures (Tul antigen, 0.1 μg/ml) are shown in the lower and upper halves, respectively. Each point represents the geometric mean counts per minute of two animals followed serially.*

encephalomyelitis (15), rickettsiae (32), and chlamydia (25). In addition, a carrier state was induced by CY in lymphocytic choriomeningitis virus infection in mice (12). When CY was administered before infection, an adverse effect was noted in a model bacterial infection with *Pseudomonas* (26) and cutaneous leishmaniasis (1). Pretreatment with CY was associated with no increase in mortality, however, in the rabies experiments (17) and with enhanced reactivity to histocompatibility antigens in other studies (21, 35). Thus, there is less information on effects of CY pretreatment than on effects of treatment after infection, and little work has been done on bacteria compared with viruses. We therefore attempted to determine the effect of CY on cell-mediated immunity to tularemia bacterial antigen in guinea pigs.

We examined the skin reactions induced in guinea pigs after immunization with killed tularemia vaccine emulsified in Freund adjuvants and the effect of CY pretreatment on these reactions. It was shown that when animals were immunized with approximately 10 μg of bacterial nitrogen tularemia vaccine in FIA, they showed skin reactivity at 1 week that looked similar to that found in previous studies after immunization with 1 μg of ovalbumin in FIA (31). Similarly, CY pretreatment caused prolonged and enhanced skin reactivity which approached that induced by tularemia vaccine in FCA (Fig. 1A). In contrast to the ovalbumin studies, at 2 weeks the skin reactivity of guinea pigs immunized with tularemia vaccine in FIA persisted. At 2 weeks in the ovalbumin system, delayed hypersensitivity could not be detected, and only Arthus reactions were found at 4 h. Immunization with tularemia vaccine in either FIA or FCA at no time induced any 4-h skin reactions, demonstrating that this antigen differed in this respect from many antigens (31). The enhancement and prolongation of skin reactions induced by pretreatment with CY did not persist when the guinea pigs, which had received the larger dose of tularemia vaccine, were skin tested 2 weeks after immunization (Fig. 1B). However, with a 10-fold-lower dose of vaccine, CY pretreatment induced persistent enhancement and duration of skin reactions even at 3 weeks (Fig. 2). Thus, the effect of CY pretreatment was most evident at the smaller dose of antigen and was less evident at an antigenic dose that was already maximally immunogenic.

Passive transfer and LT studies were undertaken to analyze these skin reactions further. The successful transfer of DTH by cells confirmed other studies establishing the cellular nature of the immune response to tularemia (7, 9, 23). Enhanced skin reactivity was transferred by cells from CY-pretreated, immunized donors compared with cells from immunized donors not pretreated with CY. This demonstrated that CY pretreatment might be a useful tool to demonstrate cellular transfer of DTH to weak antigens.

The LT studies are consistent with the cellular nature of the immune response. At all times studied after immunization, enhanced in vitro deoxyribonucleic acid synthesis was found in the presence of antigen. In contrast to guinea pigs immunized with ovalbumin in FIA, after CY, the cells from guinea pigs immunized with tularemia antigen in FIA showed enhanced transformation that persisted for 23 days (20). At this time skin reactions no longer showed a significant difference.

Studies on the relationship between DTH and resistance to tularemia in guinea pigs have previously shown no change in resistance after desensitization and loss of DTH reactivity (13). However, the effects of maneuvers or substances

that increase DTH reactivity have not been evaluated. Because of difficulties with the guinea pig challenge system to tularemia, the biological significance of the altered state of immune reactivity found in these studies will be determined in the mouse infectivity model described previously (9).

Substances, such as CY, with a selective cytotoxicity for regulator cells, enhance cell-mediated immunity and have a potential practical application in certain infectious diseases. This is particularly applicable to situations in which live vaccines can only be prepared, if at all, with great difficulty. The model system described in the guinea pig seems to lend itself well to comparative evaluation of adjuvants or immune stimulants.

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