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Cellular immunity against *Salmonella typhi* after live oral vaccine

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SUMMARY

Seventeen adult volunteers were vaccinated orally with the live attenuated *Salmonella typhi* mutant strain Ty21a. Their peripheral blood mononuclear cells were tested at different times after vaccination for direct cell-mediated activity against bacteria, employing a simple short-term *in vitro* assay. It was observed that 16/17 of the vaccinated subjects acquired the capacity to express specific cellular immunity against *S. typhi* which lasted from 15 days to at least 3 years. The effector cell of the *in vitro* antibacterial activity was preliminarily characterized as a non-adherent $T3^+$, $T8^-$, $T4^+$ lymphocyte. In parallel, mice immunized orally with *S. typhimurium* and proving resistant to reinfection were tested employing the same *in vitro* assay. Also in this case peripheral and, most important, intestinal lymphocytes were able to express cellular immunity against the agent of murine typhoid. It is concluded that administration of live oral vaccine against *S. typhi* results in the induction of specific cellular immunity which is expressed at the peripheral and, probably, also at the intestinal level.

Keywords typhoid fever oral vaccine cellular immunity

INTRODUCTION

The immune mechanisms which underline an effective protection against enteropathogenic bacteria after oral vaccination are still far from being understood. It is thought that the role of the gut-associated lymphoid tissues (GALT) is of primary importance against intestinal bacterial infections. In particular, the direct antibacterial action of the lymphoid cells which are present in high numbers within the intestinal epithelium and the lamina propria is regarded as a major first line of defence against gastroenteric infections. Since complement does not seem to be fully effective at the mucosal level, cellular immune mechanisms should be of major relevance in the expression of surveillance against intestinal infections. Therefore, it is expected that oral vaccines against enteric pathogens such as *Salmonella typhi* should mainly potentiate cell-mediated immunity. Nevertheless, only the presence of antibodies against bacterial antigens has normally been investigated to assess the effectiveness of vaccines.

A direct *in vitro* test measuring the antibacterial activity of lymphocytes from GALT and from peripheral lymphoid organs has previously been employed by us (Nencioni *et al.*, 1983; Tagliabue *et al.*, 1983, 1984a,b) to test successfully cell-mediated immunity in mice against *S. typhimurium*, the agent of murine typhoid fever. In the present study, we applied the same experimental approach, employing peripheral blood mononuclear cells from healthy young adults before and after oral

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vaccination with the live attenuated *S. typhi* mutant strain Ty21a (Germanier & Fürer, 1975). Preliminary results demonstrating the presence of a strong cellular immunity against *S. typhi* in vaccinated volunteers are reported.

MATERIALS AND METHODS

Immunization. The participants in this study were healthy volunteers of both sexes, aged between 25 and 40 years, who had not been previously vaccinated against *S. typhi.* After informed consent, blood was taken on day 0 to be tested before the vaccination. Seventeen volunteers were then vaccinated (three in 1981, three in 1982, 10 in 1983 and one in 1984) with 10^9 organisms of the *S. typhi* mutant strain Ty21a (Germanier & Fürer, 1975) (Neotyf Vaccine, Sclavo, Siena, Italy). After chewing sodium bicarbonate tablets to protect the live bacteria from gastric acidity, volunteers ingested the vaccine on day 0, +2 and +4. Blood was then collected at different times from the vaccinated subjects and from unvaccinated volunteers of the same age.

Cell separation and characterization. Mononuclear cells were separated by centrifugation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) and resuspended in RPMI 1640 medium (GIBCO-Europe, Paisley, Scotland) supplemented with 10% heat-inactivated fetal bovine serum (Sera-Lab, Crawley Down, Sussex, UK), 25 mM HEPES buffer, and 2 mM L-glutamine (hereafter referred to as test medium) right before the bacterial assay. In some experiments, 100–200 $\times$ 10^6 mononuclear cells were incubated overnight at 4°C in plastic tubes (cat. no. 2070, Falcon, Becton-Dickinson, Grenoble, France) and then washed and resuspended in 2 ml test medium to be incubated for 1 h at 37°C in 10 ml syringes containing 300 mg nylon wool (Julius, Simpson & Herzenberg, 1973). Non-adherent cells were then eluted with warm test medium. Viability of cells after this incubation exceeded 95%. Ten million non-adherent cells in 0·5 ml test medium were treated with monoclonal antibodies T3 (lot no. T307) or T4 (lot no. T407) or T8 (lot no. T808, Ortho Diagnostic Systems Inc., Raritan, New Jersey, USA) at 1:10 final dilution for 60 min on ice. After washings, cells were resuspended in 0·5 ml test medium plus 0·5 ml rabbit complement-MA (lot no. 1157, Cedarlane Laboratories Ltd, Hornby, Ontario, Canada) and then incubated 1 h at 37°C. Washed cells were resuspended in test medium without readjusting their concentrations. Viability was assessed to determine the effectiveness of antibody plus complement (C) treatments employing fluorescein diacetate/ethidium bromide staining as previously described (Tagliabue *et al.*, 1982).

Bacteria. The bacterial strains employed in this study were the pathogenic *S. typhi* strain ATCC 10749 (0–9, 12), the non-pathogenic *S. typhi* mutant strain Ty21a obtained from the Swiss Serum and Vaccine Institute Berne, Berne, Switzerland, the pathogenic *S. typhimurium* strain ATCC 14028 (0–1, 4, 5, 12) and the pathogenic *S. tel aviv* strain (0–28) from our own bacterial collection. *S. typhi* was cultivated overnight in LB Broth and *S. tel aviv* and *S. typhimurium* in tryptose broth.

Antibacterial assay. The bactericidal assay was performed as previously described in detail (Nencioni *et al.*, 1983; Tagliabue *et al.*, 1983, 1984a,b). Briefly, 10^4 bacteria were incubated with lymphoid cell suspensions at different effector cell:bacterial target (E:T) ratios for 2 h at 37°C in conical tubes. At the end of the incubation period, the remaining bacteria were plated on Petri dishes containing LB or tryptose agar. After overnight incubation, colony forming units (CFU) were counted and the percentage of antibacterial activity was calculated as 100–100 $\times$ (number of CFU of experimental tubes)/(number of CFU of control tubes without lymphocytes).

Animal studies. Ten thousand *S. typhimurium* bacteria were administered orally to BALB/c mice (obtained from Charles River, Calco, Italy), which are highly susceptible to infection with this bacterial strain (Tagliabue *et al.*, 1984b). After 60 days, the mice surviving the first challenge again received 10^6 bacteria orally. After a further 30 days the animals were sacrificed and cell suspensions from spleens and Peyer's patches were obtained to be used in the *in vitro* antibacterial tests in comparison with cells from normal BALB/c mice.

Statistical analysis. Antibacterial activity was statistically analysed by parallel-line assay after logarithmic transformation of the variables (Finney, 1971).

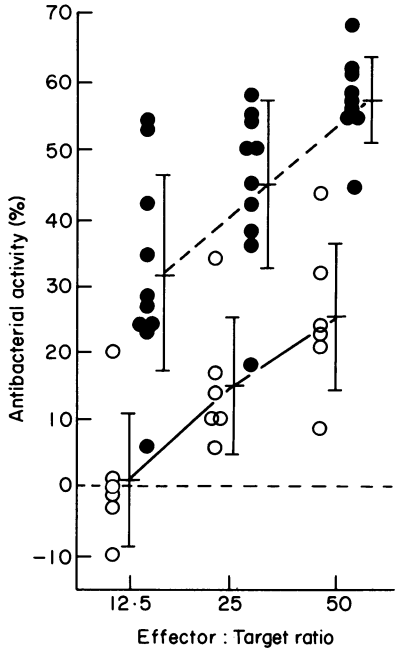

Fig. 1. Cell-mediated *in vitro* activity against *S. typhi* strain ATCC 10749 of normal (○) and vaccinated volunteers (●) 15 days after vaccination with attenuated *S. typhi* mutant strain Ty21a. Each circle represents a donor. Bars indicate mean ± s.d. Difference between slopes was statistically significant ($P \leqslant 0{\cdot}01$) as assessed by parallel line assay after logarithmic transformation of the variables (Finney, 1971).

RESULTS

Peripheral blood mononuclear cells from normal healthy adults possess low natural antibacterial activity when tested *in vitro* against pathogenic *S. typhi* strains (Fig. 1). After receiving the live oral vaccine *S. typhi* mutant strain Ty21a, the *in vitro* antibacterial activity was strongly increased in 9/10 volunteers. Figure 1 shows the activity of vaccinated subjects 15 days after the last vaccine administration tested in parallel with unvaccinated volunteers (results are a pool of three experiments). The activity of the same subjects before and after oral vaccination was then tested and results reported in Fig. 2 show the striking increase in antibacterial activity in four volunteers after oral vaccination. The antibacterial activity of cells from vaccinated subjects appeared to be quite specific and long lasting. In fact, no increase of the natural activity against *S. tel aviv*, a Salmonella

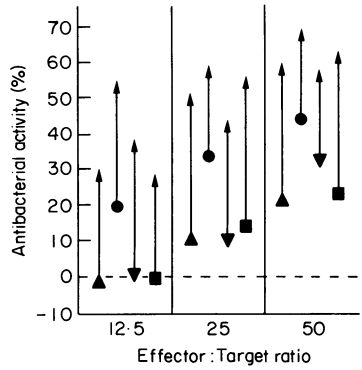

Fig. 2. Cell-mediated *in vitro* activity against *S. typhi* strain ATCC 10749 of four volunteers tested individually immediately before (▲, ●,▼,■) and 15 days after vaccination (arrows indicate increase in activity).

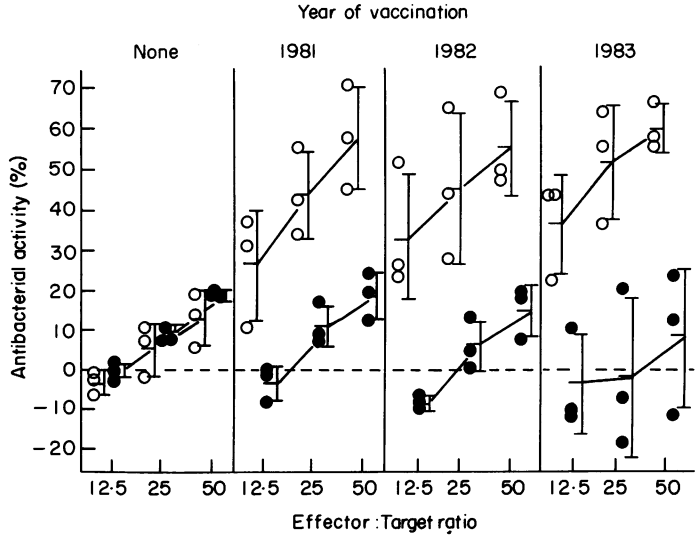

Fig. 3. Cell-mediated *in vitro* activity against *S. typhi* (○) and *S. tel aviv* (●) of normal and vaccinated subjects at different times after administration of live oral vaccine. Bars indicate mean ± s.d. Differences between slopes of unvaccinated and vaccinated subjects assessed as in Fig. 1 were statistically significant ($P \leqslant 0{\cdot}01$) with *S. typhi* targets and not significant with *S. tel aviv* targets.

strain with somatic antigens differing from those of *S. typhi*, could be observed after the volunteers received the *S. typhi* mutant strain Ty21a (Fig. 3). In contrast, the *in vitro* activity against *S. typhi* in these subjects was increased and remained high for at least 3 years after vaccination (Fig. 3).

Since previous studies with murine cells in the above described antibacterial *in vitro* assay revealed that the effector cell is a lymphocyte (Nencioni *et al.*, 1983; Tagliabue *et al.*, 1983, 1984a), a preliminary characterization of the human effectors was here performed. Mononuclear leukocytes from peripheral blood of normal and vaccinated subjects were passed on nylon wool columns to eliminate most of the adherent monocytes and B cells (Julius *et al.*, 1973). Then the effector populations were treated with monoclonal antibodies against T cell subsets and C. Figure 4 shows that the oral vaccine was able to induce an eight-fold increase in the antibacterial activity as judged by the shift towards lower E : T ratio values, and that in both cases T3 and T4, but not T8 antibodies, abolished the activity in presence of C.

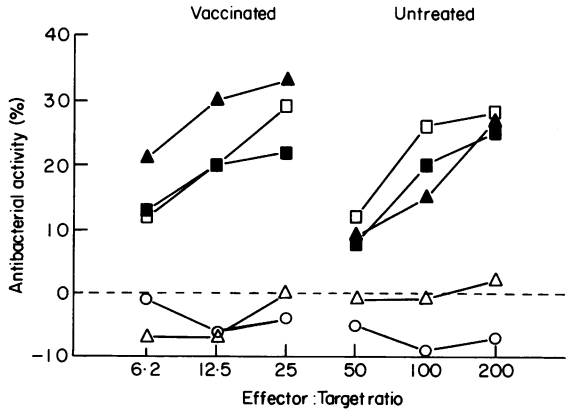

Fig. 4. Cell-mediated *in vitro* activity of nylon wool non-adherent mononuclear blood leucocytes from one normal and one vaccinated volunteer before (□) and after treatment with C (■), T3+C (○), T4+C (△) and T8+C (▲).

Table 1. *In vitro* cell-mediated immunity against *S. typhimurium* of normal and orally immunized BALB/c mice.

		Antibacterial activity (%)			
Mice	Cell source	25*	50	100	200
Normal	spleen	−4	6	11	11
Immunized†	spleen	27	35	43	61‡
Normal	Peyer's patches	−1	7	13	18
Immunized†	Peyer's patches	17	25	36	43‡

* E:T ratio.

† As described in Materials and Methods.

‡ $P \leqslant 0{\cdot}01$ versus corresponding normal group assessed by parallel line assay after logarithmic transformation of the variables (Finney, 1971).

In a further attempt to establish an experimental model which would allow us to compare *in vivo* resistance to typhoid fever and *in vitro* activity against the bacteria responsible for the infection, BALB/c mice were orally immunized with *S. typhimurium*. After the observation that they were resistant to oral reinfection with bacteria at doses several fold higher than LD_{50}, cells from spleens and Peyer's patches were employed for the *in vito* assays. Table 1 shows that the mice surviving infection had significantly higher antibacterial activity than normal mice. This acitivity was evident with lymphocytes from a peripheral organ such as the spleen and, more important, with cells from Peyer's patches, which are part of the gut-associated lymphoid system.

DISCUSSION

In a recent controlled field trial held in Alexandria, Egypt, in which a total of 32,388 children participated, live oral *S. typhi* Ty21a vaccine was observed to reduce the incidence of typhoid fever from 4·9 to 0·2 cases per 10,000 children per year (Wahdan *et al.*, 1982). The overall conclusion was that this oral vaccine is protective for a period of at least 3 years. It was also hypothesized that its effectiveness could be due to the stimulation of the intestinal immune responses which are more successfully activated by the live bacteria introduced via the oral route (Woodward & Woodward, 1982).

In an attempt to analyse the mechanisms of action of this vaccine and to provide a tool to monitor the extent and duration of its effectiveness, we performed a preliminary study with 17 vaccinated volunteers, following an experimental approach previously applied with success to the analysis of cell-mediated immune responses in mice (Nencioni *et al.*, 1983, Tagliabue *et al.*, 1983, 1984a,b). The results obtained clearly showed that the vaccinated volunteers acquired a specific cellular immunity against *S. typhi* which can be determined with this *in vitro* test for at least 3 years, i.e. the period of time during which the protection induced by the vaccine has been proved to last (Wahdan *et al.*, 1982).

According to previous results in mouse systems (Nencioni *et al.*, 1983; Tagliabue *et al.*, 1983, 1984a,b), a preliminary characterization of the human effector cell of the antibacterial activity revealed that this might be a lymphocyte, probably of a T-cell subset. This would reasonably rule out that phagocytosis might be the antibacterial mechanism employed by the effector cells *in vitro*. Since also bacterial aggregation on lymphocyte surfaces seems unlikely to play a role in our system (careful microscopic controls were performed on the colonies growing on agar after plating vigorously vortexed cell suspensions), the mechanism at the basis of the antibacterial activity remains to be elucidated. It seems of interest that pretreatments of the effector cells with anti-IgA antibodies are capable of blocking the activity of lymphocytes from both normal and vaccinated volunteers (Nencioni *et al.*, unpublished observation). Thus, it can be suggested that, according to

previous results in mice (Tagliabue *et al.*, 1983, 1984a), also in this human system a mechanism of antibody-dependent cellular cytotoxicity expressed by lymphocytes and IgA might be important.

Taken all together, these results indicate that cell-mediated immunity against enteropathogenic bacteria after administration of an effective oral vaccine can be measured with a direct, simple test. Further studies are of course necessary to prove the hypothesis that the cellular activity observed with peripheral lymphocytes reflects that of intestinal cells. However, employing a mouse model of typhoid fever, which is similar though not identical to the human one, a correlation between peripheral and intestinal cell-mediated responses was observed. In fact, after oral infection with *S. typhimurium* the surviving mice became resistant to a second challenge with bacteria and developed in parallel a strong antibacterial activity measurable *in vitro* with both the peripheral and intestinal lymphocytes.

In conclusion, the possibility of measuring cell-mediated antibacterial activity in orally vaccinated subjects may find at least two applications. The first is to use this experimental approach as a tool to better analyse the mechanism of immune responses against gastrointestinal infections. The second is to employ this assay to assess the degree and length of protection induced by the oral vaccine in high-risk groups such as laboratory personnel, travellers, foreign-based military personnel, missionaries and physicians operating in endemic typhoid areas in an attempt to avoid vaccine failures (Hirschel, 1983).

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