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Comparative Analysis of Immunological Responses to Oral (Ty21a) and Parenteral (TAB) Typhoid Vaccines

RAFFAELE D'AMELIO,[1]* ALDO TAGLIABUE,[2] LUCIANO NENCIONI,[2] ARMANDO DI ADDARIO,[3] LUIGI VILLA,[2] MARIO MANGANARO,[4] DIANA BORASCHI,[2] SOCCORSA LE MOLI,[1] ROBERTO NISINI,[1] AND PAOLO M. MATRICARDI[1]

Aeronautica Militare, D.A.S.R.S., Rep. Medicina, Gruppo di Igiene ed Immunologia, Pratica di Mare,[1] Centro Studi Sanita' Esercito,[3] and Clinica Malattie Tropicali e Infettive, Universita' "La Sapienza,"[4] Rome, and Sclavo SpA, Lab. Immunofarmacologia, Centro Ricerche, Siena,[2] Italy

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The clinical and immunological responses to typhoid vaccination with parenteral (TAB) and oral (Ty21a) vaccines in two groups of 30 adult male subjects were studied. Parameters monitored included specific anti-*Salmonella typhi* cell-mediated immunity and total and specific antilipopolysaccharide fecal immunoglobulin A (IgA) titers in Ty21a-vaccinated subjects. Peripheral blood lymphocyte antibacterial activity was significantly increased only in Ty21a-vaccinated subjects. Serum arming activity and results of human $F(ab')_2$ anti-IgG and -IgA inhibition tests suggest antibody-dependent cellular cytotoxicity mediated by IgA in those vaccinated with Ty21a. Interestingly enough, the cells of TAB-vaccinated subjects were able to mediate IgG-dependent cellular cytotoxicity, as was observable from the results of blocking experiments. Moreover, total and specific antilipopolysaccharide fecal IgA levels were observed to be significantly increased with Ty21a, up to 8 months post-vaccination schedule. An early-onset, transitory increase in serum IgM rheumatoid factor was also found, exclusively in subjects treated with TAB, and was no longer detectable on day 240. Ty21a was well tolerated and free of side effects, whereas 65% of subjects administered TAB reported fever, headache, malaise, and local tenderness at the injection site. Our data show that the two typhoid vaccines induce different cell-mediated specific immune responses. The role of these responses in protection against *Salmonella* infection, however, requires further investigation.

Typhoid fever is still a major infectious disease in the developing world, and it also affects Mediterranean countries. The mechanisms of immunization against *Salmonella typhi* are not yet completely clear. Observations made of experimental animals and patients with typhoid fever seem to assign a more important role to cell-mediated than to humoral immunity. A useful model for investigating the immunological problems connected with the response during natural infection is the study of human volunteers after vaccination. Indeed, this has been done in the past with TAB vaccinees (3, 10, 13). Although the traditional TAB vaccine confers satisfactory and substantial protection (21), its administration provokes severe side reactions.

Recently, a live attenuated oral vaccine (Ty21a) prepared from enzyme-deficient organisms (6) has been introduced. Epidemiological studies have demonstrated the clinical safety and efficacy of this type of vaccine (7, 20). The aim of this study was to compare the humoral and cell-mediated specific and nonspecific immune responses induced by the oral versus parenteral vaccine in groups of healthy adult male subjects. In addition, considering that the route of administration of Ty21a vaccine parallels that of natural infection, it can also be hypothesized that this represents the best experimental model to gain some insight into the immune response during typhoid infection.

MATERIALS AND METHODS

Subjects. Two groups of 30 healthy male subjects, ages between 16 and 22, were studied. The first group was treated subcutaneously with TAB vaccine (ISI, Milan, Italy) on days 0 and 28. The second group was treated with live oral vaccine (Ty21a; Vivotif, Berne, Switzerland, or Neotyf, Sclavo, Italy) on days 0, 2, and 4 with 10^9 organisms per dose of the *S. typhi* mutant strain Ty21a (6). Informed consent was obtained in all cases. The criteria for admission to the study protocol were the absence, or presence of very low levels, of antilipopolysaccharide (anti-LPS) antibodies ($\geq$1.25 ng of specific antibodies per well; see below for enzyme-linked immunosorbent assay [ELISA]) in the serum and the absence of *S. typhi* in fecal cultures.

Study protocol. Blood specimens were drawn on days 0, 15, 30, and 240 from the beginning of the vaccination schedule. The following parameters were evaluated in the serum at these times: immunoglobulin G (IgG)-, IgA-, and IgM-rheumatoid factors (RF) and anti-LPS IgG, IgA, and IgM.

Heparinized peripheral venous blood was used for total and differential leukocyte counts, for the monitoring of CD3-, CD4-, and CD8-positive lymphocytes, and for specific cell-mediated antibacterial assays. Fecal samples were collected from the Ty21a-vaccinated group on days 0, 30, and 240, and total and specific anti-LPS IgA levels were evaluated. On day 30, fecal cultures for *S. typhi* were repeated in the same group.

RF. Blood specimens were allowed to coagulate and were stored in separate samples at −70°C within 3 h from the time of sample collection. IgM- and IgA-RF were determined by an ELISA in 96-well polystyrene microplates by using rabbit IgG (10 μg/ml) as antigen. Samples were diluted 1:50, and the RF were detected with an alkaline phosphatase-conjugated $F(ab')_2$ goat anti-human IgA or IgM (Zymed). IgG-RF was assessed in sera previously reduced with 0.4 M 2-

* Corresponding author.

mercaptoethanol (Calbiochem-Behring, La Jolla, Calif.) and alkylated with 0.4 M iodacetamide (Sigma Chemical Co., St. Louis, Mo.). In this case, RF was detected by a rabbit alkaline phosphatase-conjugated $F(ab')_2$ anti-human IgG prepared in our laboratory from the same animal which had furnished the IgG antigen, before immunization with human IgG, in order to minimize the background as much as possible. Four internal laboratory positive standard reference sera were included in each plate together with 10 negative sera. Results were expressed in micrograms per milliliter.

ELISA for anti-LPS antibodies. Flat-bottom polystyrene microtest plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with LPS-W *S. typhi* 0901 (Difco Laboratories, Detroit, Mich.) at a concentration of 100 μg/ml in carbonate-bicarbonate buffer (0.05 M, pH 9.6).

To minimize nonspecific adsorption of serum protein to the plastic, the wells were incubated with a blocking solution consisting of 2% bovine serum albumin in phosphate-buffered saline for 2 h at 37°C. Serum samples were added at dilutions of 1:50 for detection of IgA and IgM and 1:100 for detection of IgG.

After incubation, 100 μl of class-specific goat anti-human immunoglobulin conjugated with alkaline phosphatase (anti-IgA, lot 23069; anti-IgG, lot 25808; anti-IgM, lot 27150; Organon Teknika, Malvern, Pa.) was added to each well. The enzyme-conjugated antibodies used in the assay had previously been titrated against chromatographically purified human antibodies (Organon Teknika) and were diluted 1:500 for IgA, 1:1,500 for IgG, and 1:1,000 for IgM.

After incubation at 37°C, plates were washed and 100 μl of *p*-nitrophenyl phosphate substrate (Sigma), 1 mg/ml in 1 M diethanolamine (pH 9.8)–1 mM $MgCl_2$, was added to each well.

Controls for each plate included wells with serum samples, but no antigen, and wells with antigen and affinity-purified human IgA, IgG, and IgM used for the standardization assay but without serum samples. This technique allowed the detection of as little as 1.25 ng of specific antibody per well. Each serum sample was tested in duplicate, and absorbance values were averaged.

Fecal IgA. Feces (10 g) from each subject were collected and centrifuged, and supernatants were diluted 1:10 with phosphate-buffered saline. Total IgA levels were determined by a nephelometric assay. Specific IgA anti-LPS levels were determined by ELISA method as described above after dilution of all supernatants at 10 μg of total IgA per ml.

Cellular studies. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized venous blood specimens, layered on Ficoll-Hypaque density gradient, and centrifuged at 900 × *g* for 20 min. After centrifugation, PBMC were collected from the interface. Cell viability, as checked by trypan blue exclusion, was always greater than 95%.

Labeling of lymphocytes with monoclonal antibodies. PBMC were incubated first with monoclonal antibodies OKT3-4-8 (Ortho Pharmaceutical Corp., Raritan, N.J.) and then with fluorescein isothiocyanate-labeled goat anti-mouse IgG (Meloy Labs, Mississauga, Ontario, Canada). The percentage of fluorescence-positive cells was determined by means of a Leitz UV microscope equipped with vertical illumination.

Antibacterial assay. The antibacterial assay was performed as described in detail with *S. typhi* Ty2 used as target (12, 16, 19). Briefly, bacteria were placed in 15-ml conical tubes together with either medium or appropriately diluted antibodies and were centrifuged at 1,300 × *g* for 10 min at 4°C. The cell suspensions were then added to bacteria at different effector-to-target (E:T) ratios, and the tubes were again centrifuged at 500 × *g* for 5 min at 4°C. To maintain optimal proportions of the reactants, the final volume of the mixture was limited to 0.3 ml, consisting of 0.1 ml each of bacterial suspension, medium, and effector cells. The experimental and control tubes, which contained bacteria and medium but not cells, were then incubated at 37°C for 2 h. At the end of the incubation period, the pellets were suspended, the volume was brought to 1 ml, and appropriately diluted portions were plated on petri dishes containing agar tryptose. After overnight incubation, CFU were counted. Usually 100 or 200 CFU were scored per dish in control groups. Duplicate tubes were set up for each experimental group, and two petri dishes were prepared for each tube. The percentage of antibacterial activity was calculated as follows: percent antibacterial activity = 100 − [100 × (number of CFU in experimental tubes/number of CFU in control tubes without cells)].

For blocking studies, the following antibodies were used: $F(ab')_2$ fragments of goat anti-human serum IgA (lot 23119, Organon Teknika) and goat anti-human IgG (lot 24063, Organon Teknika) obtained from affinity chromatography-purified antibodies. Effector cells were pretreated with $F(ab')_2$ fragments for 1 h at 4°C and then washed twice in phosphate-buffered saline.

Statistical analyses. Statistical analyses were generally carried out by one-way analysis of variance modified for randomized groups or Student's paired *t* test. For antibacterial activity, results are expressed as the mean, and the standard error is not reported because it was usually less than 10%. These results were statistically analyzed by parallel line assay (5) after logarithmic transformation of the variables.

RESULTS

Humoral and cellular immunity were assessed in healthy male volunteers after oral Ty21a or parenteral TAB vaccine.

Humoral immunity. An increase in the concentration of IgG anti-LPS was observed in both the orally and parenterally vaccinated volunteers 30 days after immunization, whereas serum IgA anti-LPS augmented slightly only after oral Ty21a vaccine. On day 240, the serum concentration of the specific antibodies did not differ from that assessed before vaccination in either group, whereas IgG seroconversion was still detectable in a significant number of volunteers after TAB vaccine (data not shown).

A remarkable and very significant increase in IgM-RF was observed in the TAB-vaccinated group on days 15 and 30, whereas no major modification could be detected in Ty21a vaccinees (Fig. 1). No significant modification of other RF isotypes was observed in either group. Similarly, total serum immunoglobulin levels (and CIC) remained unaltered in both groups during the observation period (data not shown).

Fecal IgA. A strong increase in levels of fecal IgA against LPS was observed 30 and 240 days after Ty21a vaccine (Fig. 2). In parallel, total IgA levels were found to be significantly increased in the feces by oral vaccine on days 30 and 240.

Cellular immunity. No significant variations were observed either in the percent or in the absolute number of CD4- and CD8-positive PBMC, so that the T4/T8 ratio remained virtually unchanged on both days 15 and 30 versus day 0.

By using a direct antibacterial in vitro assay previously shown to be effective in experimental and clinical systems

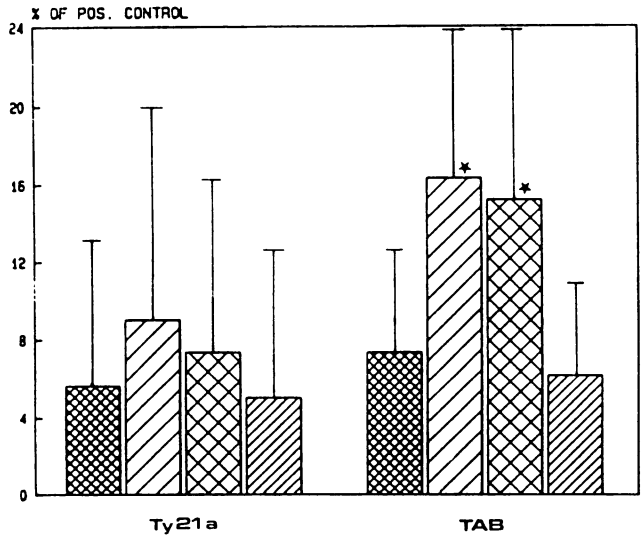

FIG. 1. IgM-RF at 0 (▦), 15 (▨), 30 (▩), and 240 (▨) days form the first administration of TAB or Ty21a in two groups of 30 subjects each. An asterisk indicates $P < 0.01$ versus value at day 0.

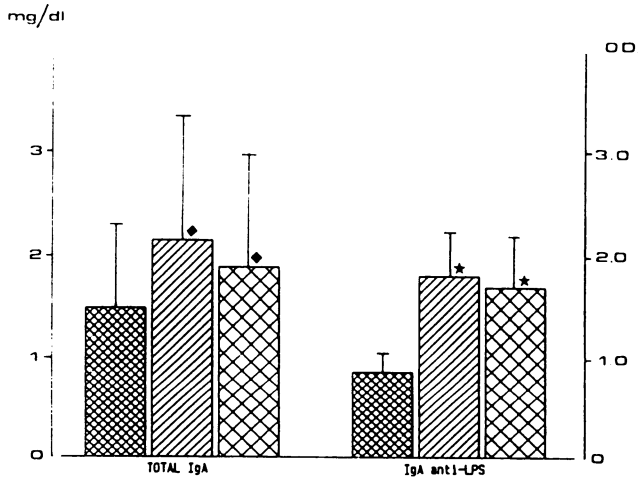

FIG. 2. Total and anti-LPS fecal IgA in 30 subjects at 0 (▦), 30 (▨), and 240 (▩) days from oral administration of Ty21a. $P < 0.01$ (⋆) and $P < 0.05$ (◆) versus value at day 0 are indicated.

(12, 17, 18), a comparison was made between oral Ty21a and parenteral TAB vaccines in relation to their capacity to induce cellular immunity against *S. typhi* and other *Salmonella* strains. At 30 days after Ty21a vaccination, a statistically significant increase in direct antibacterial activity was observed against *S. typhi* and cross-reacting *S. paratyphi* A and B, but not against antigenically unrelated *S. paratyphi* C, whereas TAB vaccine did not induce this response (Table 1). When this assay was performed 240 days after vaccination, it was observed that Ty21a vaccinees, but not TAB volunteers, still demonstrated strongly enhanced cellular immunity against *S. typhi* (Table 1).

Since previous experiments (12, 17, 18) have shown that direct antibacterial activity, as assessed by this in vitro assay, is antibody dependent, the arming capacity of sera on normal PBMC from Ty21a and TAB vaccinees was tested. Serum samples taken 30 days after Ty21a vaccination strongly increased the anti-*S. typhi* activity of PBMC from normal donors, whereas nothing was observed when serum samples from TAB vaccinees taken after 30 days were used (Table 2).

Blocking tests with anti-human IgA and IgG were performed by pretreating the effector cells before the in vitro assays. As a confirmation of previous studies (19), it was observed that the $F(ab')_2$ fragment of anti-human IgA completely blocked natural as well as Ty21a-induced antibacterial activity (Table 3). Interestingly, the same did not occur after TAB vaccination, but pretreatment with $F(ab')_2$ fragments of anti-human IgG completely abolished the antibacterial activity of TAB vaccinees (Table 3).

Clinical data. None of the subjects treated with live attenuated oral vaccine reported any noteworthy side effects, whereas over 65% of those given TAB had such complaints as local tenderness at the injection site, general malaise, fever, and headache.

DISCUSSION

Typhoid fever is still a major world health problem, with millions of cases occurring each year in developing countries. Control of typhoid has primarily resulted from improved environmental hygienic measures rather than the use of a safe and effective specific vaccine. The traditional vaccine, injected subcutaneously and consisting of killed *S. typhi* organisms, enhances human resistance to typhoid but provokes severe side effects. A new live attenuated oral vaccine prepared with an enzyme-deficient organism has recently been licensed and has resulted in long-lasting (nearly 3 years) and effective protective immunity, according to immunological and epidemiological studies (16, 19, 20).

TABLE 1. Cell-mediated immunity against *Salmonella* infection

Vaccination	Time (day)	% Antibacterial activity against:															
		S. typhi				*S. paratyphi* A				*S. paratyphi* B				*S. paratyphi* C			
		6[a]	12	25	50	6	12	25	50	6	12	25	50	6	12	25	50
Ty21a	0	−25	−8	0	8	−20	−13	−2	1	−12	−3	−3	−1	−3	−10	−3	7
	30	8	25	37	53[b]	12	22	37	42[b]	6	16	13	18[b]	−16	2	−8	−9
	240	19	19	23	43[b]	ND[c]	ND[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
TAB	0	−5	−8	1	15	−5	3	4	12	−7	1	3	2	−15	−15	−15	11
	30	−3	1	9	17	−8	8	9	16	−11	−6	−13	8	−7	−10	−19	8
	240	1	6	10	15	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Effector/target ratio.
[b] $P < 0.01$ versus value for day 0 control (curves corresponding to the different effector/target ratios are compared).
[c] ND, Not done.

TABLE 2. Arming activity of serum samples from volunteers before and after vaccination with Ty21a and TAB

Vaccine	Time (day)	% Antibacterial activity at E:T[a]:		
		12	25	50
None		12	5	19
Ty21a	0	13	10	17
Ty21a	30	43[b]	41[b]	49[b]
TAB	0	12	4	4
TAB	30	8	12	14

[a] E:T, Effector/target ratio. Effector cells were normal PBMC in each experiment.
[b] $P < 0.01$ versus normal PBMC.

TABLE 3. Effects of pretreatment with anti-human (anti-h) IgA antibodies on the antibacterial activity of PBMC from normal donors and vaccinees

Expt no. and donor group	Treatment	% Antibacterial activity at E:T[a]:			
		12	25	50	100
1					
Healthy	None	−2	5	0	10
	$F(ab')_2$ anti-h IgA	−20	−16	−24	−18[b]
	$F(ab')_2$ anti-h IgG	−18	−7	−16	7
TAB	None	−1	0	4	20
	$F(ab')_2$ anti-h IgA	16	11	16	16
	$F(ab')_2$ anti-h IgG	2	−1	−8	−2[b]
2					
TAB	None	−4	10	3	18
	$F(ab')_2$ anti-h IgA	15	18	22	25
	$F(ab')_2$ anti-h IgG	4	−11	−3	−18[b]
Ty21a	None	6	0	15	19
	$F(ab')_2$ anti-h IgA	9	4	7	−6[b]
	$F(ab')_2$ anti-h IgG	22	22	34	34

[a] E:T, Effector/target ratio.
[b] $P < 0.01$ versus value for corresponding control (curves corresponding to the different effector/target ratios are compared).

In accordance with literature data concerning clinical tolerance, in this study Ty21a has proved to be completely safe, whereas TAB induced marked local phenomena and systemic symptomatology in over 65% of vaccinated subjects, mainly after the second injection.

Immunological monitoring was done by evaluating cellular and humoral specific and nonspecific alterations. Specific cell-mediated immunity, assessed in PBMC by a simple short-term in vitro assay, involved the appearance of a strong specific immune response against *S. typhi* on day 30 and also against *S. paratyphi* A and B in subjects immunized with Ty21a, whereas a complete absence of response was observed in TAB-vaccinated subjects in this test. Cell-mediated activity against *S. typhi* was still present on day 240 in Ty21a vaccinees (but did not appear in subjects taking TAB). Thus, these results confirm our previous experience (16, 19) and clearly indicate that the immune responses induced by oral and parenteral vaccines differ greatly. Taking into account the presence of fecal IgA which was observed after Ty21a vaccination in this and a previous study (1), it might be suggested that the induction of specific antibodies of IgA isotype against *S. typhi* and perhaps cross-reacting bacterial species is the primary event of vaccine-induced immunity. This may then lead to a mucosal response mediated by secretory IgA and/or local and systemic IgA-driven cellular immunity. Since lymphocytes of the CD4 subset have been shown to be the cellular arm of IgA-dependent antibacterial activity (18, 19), the observation reported here that $CD4^+$ cell numbers are not increased in Ty21a vaccinees could further indicate that the vaccine action resides mainly in a potentiation of the humoral arm of lymphocyte antibacterial activity. A recent study by Levine and co-workers (8) concerning two attenuated auxotrophic mutant strains of *S. typhi* tested as vaccine candidates has also shown the presence of antibody-driven cellular immunity. All these results taken together suggest that this is a general immune mechanism evoked by oral vaccines.

As far as the parenteral vaccine is concerned, it was shown here that TAB is not able to induce cellular immunity beyond natural values (18), nor is it capable of generating arming antibodies, as was observed with Ty21a. It is, however, of great interest that blocking experiments showed that the low cellular immunity measurable after TAB vaccination is mediated by IgG rather than by IgA, as is the case in natural immunity (18) and oral vaccine-induced immunity (19). A major role of IgG after parenteral vaccination is widely expected, and the animal models for salmonellosis have demonstrated the role of complement and/or phagocytic cells (4, 9, 14). Indeed, TAB vaccine has shown efficacy for decades in millions of people, even though the extent of its action may be debatable. The preliminary observations reported here that the administration of a parenteral vaccine would substitute an IgG-driven response for IgA-dependent antibacterial activity stimulate further investigation.

A first, rather evident effect of qualitative change in antityphoid immunity by TAB vaccine might be the appearance of RF. In fact, IgM-RF was observed in subjects vaccinated with TAB on days 15 and 30, whereas no increase could be detected in IgG-, IgA-, or IgM-RF in Ty21a vaccinated subjects. This increase was transient, however, and was no longer present on day 240, similar to results in a case observed by us of subjects vaccinated against hepatitis B with a vaccine of French manufacture (2). The significance of such an increase is uncertain, in the light of recent results of Nemazee (11) on the induction of RF in mice after the secondary response and the enhancing effect of such RF in antigen-antibody reaction, as stated by the same author (11). This might be either a physiological response of the immune system to the formation of CIC, with a regulatory role, or a sign of B-cell polyclonal activation.

In conclusion, we have shown here that in addition to the lack of strong side effects with Ty21a, contrary to what occurs with TAB, marked differences exist between the two in evoking a specific immune response. It is important to further elucidate advantages and disadvantages of the two types of immunization in consideration of the continuous effort to provide new, and hopefully more effective, vaccines against *S. typhi* such as the Aro mutant (8) in the parenteral anti-Vi vaccines (15).

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