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Stimulation of Antibacterial Vaccination in Mice by Polyadenylic Acid: Polyuridylic Acid Complex

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The effect of polyadenylic acid:polyuridylic acid complex (poly A:U) on immunization was investigated in mice vaccinated by killed *Brucella melitensis* cells suspended in incomplete adjuvant or in saline. Addition of 300 μg of poly A:U to vaccines rendered 3×10^8 *B. melitensis* cells in saline as immunogenic as 3×10^{11} cells in oil adjuvant against a severe *B. abortus* challenge. In the mouse and *Brucella* system, poly A:U exerts an adjuvant effect on immunity but does not stimulate production of circulating antibodies that could be demonstrated at the time of autopsy.

Treatment by nontoxic polyadenylic acid: polyuridylic acid oligonucleotide complex (poly A:U) demonstrated a pronounced stimulation of antibody formation (1-6, 9, 11).

We thought it of interest to evaluate adjuvant effects of poly A:U treatment on specific immunization of mice against *Brucella abortus* challenge. Indeed, mice were unable to produce specific circulating antibodies after acute or chronic *B. abortus* infection (13). Therefore, if stimulation of *Brucella* vaccines by poly A:U was demonstrated it may add to knowledge on the mode of action of these homopolymers and may prompt assays on the practical use of poly A:U as adjuvant to vaccines.

A preliminary experiment reported here introduces the first study on the direct action of poly A:U on vaccine stimulation.

MATERIALS AND METHODS

All tests involved an appraisal of mouse immunity based on counts of *B. abortus* colonies per 1 g of spleen from animals challenged 42 days after vaccination and sacrificed 11 days after challenge with virulent *B. abortus* strain 544.

Aseptically removed spleens were weighed and ground in a Potter apparatus. The homogenate from each spleen was suspended in sterile saline to obtain 1×10^{-1} to 1×10^{-4} (w/v) dilutions. Known amounts of undiluted and diluted spleen homogenates were plated onto Trypticase soy agar (BD-M, Lyon, France) by using calibrated microsyringes and then incubated at 37 C in a 10% carbon dioxide atmosphere. The means of the number of colonies of *B. abortus*, developed from 1 g of spleen tissue, were calculated, together with standard errors (SE), for each group of mice. Also, 0.2 g each of liver and kidneys was plated onto Trypticase soy agar, and the results were scored as being positive or negative for *B. abortus*. All mice, including control unvaccinated animals, were challenged with 2×10^5 viable *B. abortus* 544 cells, corresponding to 100 median infective doses (100 ID_{50}).

We tested two vaccines: (i) Formalin-killed *B. melitensis* strain H-38 in oil adjuvant (12; abbreviated: H-38 vaccine), and (ii) Formalin-killed *B. melitensis* strain H-38 suspended in saline (abbreviated: saline vaccine). Mice were vaccinated by subcutaneously injecting 0.2 ml of the chosen vaccine. We employed female CD-1 (Caesarean-delivered, Charles River) mice, weighing about 20 g. Each group, including unvaccinated control mice, was composed of 14 or 15 mice (Table 1). Unvaccinated mice, as control of *B. abortus* challenge, were included in group 1. In group 2, mice were injected with a 1:10 dilution of the saline vaccine, thus receiving 3×10^8 Formalin-killed cells. In group 3, mice were vaccinated (3×10^9 killed cells) with saline vaccine. Mice vaccinated with a 1:10 dilution of H-38 vaccine composed group 4; in group 5, mice were injected with full H-38 vaccine, thus receiving 3×10^{11} *B. melitensis* cells in oil adjuvant. Mice in groups 6 to 9 were respectively vaccinated as in group 2 to 5, and 300 μg of poly A:U per mouse dose was added to the appropriate vaccines (Table 1). Poly A:U was prepared as follows. Polyadenylic and polyuridylic acids were obtained from Miles Laboratories, Elkhart, Ind. To 3 mg of poly A (batch N° 64) and 3 mg of poly U (batch N° 62) in a test tube, 5 ml of saline was added. After standing overnight at 4 C, the solution was kept in an 80 C water bath for 10 min and then cooled to room temperature. Formation of a double-stranded complex was verified by measuring hyperchromic change at 260 nm. Three serological tests were employed. Tube agglutination (TA) tests and complement fixation (CF) tests were performed in accordance to Joint FAO/WHO Expert Committee Recommendation (11) following techniques already described (14).

Passive hemagglutination test involved coating of a soluble *B. abortus* antigen onto sheep red blood cells through chromium chloride (15). Blood samples were obtained by aseptic transorbital puncture of the sphenoid sinus of mice before autopsies.

RESULTS

Effects of poly A:U treatment in mice immunized with adjuvant vaccine. Injection of 300 μg of poly A:U together with H-38 vaccine produced only a slight modification of the response obtained after immunization of CD-1 mice by H-38 vaccine alone (Table 1, group 9). However, mice were protected against *B. abortus* challenge when vaccinated by 1:10 diluted H-38 vaccine containing 300 μg of poly A:U per mouse dose. Such a protection was comparable to that one achieved following the use of undiluted H-38 vaccine alone (Table 1, group 8). Furthermore, colony counts of spleen cultures from poly A:U-treated mice, vaccinated by 1:10 diluted H-38 vaccine, then challenged, fell within a narrower range, thus permitting the evaluation of an SE. A similar calculation was not feasible from the data for mice vaccinated with 1:10 diluted H-38 vaccine alone where two positive spleens displayed colony counts of 8×10^4 and 1×10^5, respectively, unrelated to those counts that have been made from six other spleens which evidenced homogeneous data (272 ± 49 live *B. abortus* cells per g [Table 1, group 4]).

Effects of poly A:U treatment in mice immunized with saline vaccine. Poly A:U increased protection against *B. abortus* challenge in mice vaccinated either by 3×10^9 or by 3×10^8 *B. melitensis*-killed cells in saline (Table 1, groups 6 and 7). Counts of *B. abortus* colonies in spleen cultures of poly A:U-treated mice, vaccinated by saline vaccine, were similar to those counts obtained in strain H-38-vaccinated mice. As in mice vaccinated by diluted H-38 vaccine, poly A:U treatment suppressed the large differences in *B. abortus* counts in spleen cultures that were observed from those mice vaccinated with saline vaccine alone (Table 1, groups 2 and 3).

Effects of poly A:U treatment, vaccines, and challenge on specific circulating anti-Brucella antibodies. *B. abortus* infection was unable to induce specific, free antibodies that could be demonstrated 11 days after challenge by TA, CF, and *p*H tests in control unvaccinated mice (Table 2). Two injections of *Brucella* antigens (vaccine-associated or not with incomplete adjuvant and challenge, as a booster 42 days later) resulted in a modest production of these specific antibodies in sera from 7 out of 57 mice, sampled 11 days after the second antigen injection. Addition of 300 μg of poly A:U to saline vaccines or to H-38 vaccines did not significantly modify the induction of freely circulating *Brucella* antibodies, as only 4 out of 57 mice were found to be serologically positive (Table 2). Of 11 mice sero-

TABLE 1. *Effects of 300 μg of poly A:U per mouse dose added to Brucella vaccines upon immunization in mice against B. abortus challenge*

Treatment	I/T[a]	*B. abortus* per gram of infected spleen (avg + SE)[b]
None		
Control of *B. abortus* challenge	14/14	$4.5 \times 10^6 \pm 350{,}000$ (14 mice)[c]
Saline vaccines alone		
1:10 saline vaccine	10/14	2×10^5 (1 mouse) 8×10^4 (1 mouse) $1{,}611 \pm 501$ (8 mice)
Saline vaccine	6/15	1×10^5 (1 mouse) $1{,}317 \pm 475$ (5 mice)
Adjuvant vaccines alone		
1:10 H-38 vaccine	9[d]/14	1×10^5 (1 mouse) 8×10^4 (1 mouse) 272 ± 49 (6 mice)
H-38 vaccine	6/14	59 ± 14 (6 mice)
Poly A:U added to		
1:10 saline vaccine	9[e]/14	168 ± 19 (7 mice)
Saline vaccine	8/14	116 ± 12 (8 mice)
1:10 H-38 vaccine	6/14	55 ± 6 (6 mice)
H-38 vaccine	4/15	89 ± 5 (4 mice)

[a] I/T, Number of *B. abortus*-infected mice, on total number of mice in the group.
[b] Avg ± SE, Average number of *B. abortus* colonies ± standard error.
[c] Numbers of infected mice, to evidence dispersed bacterial counts in groups 2, 3, and 4.
[d] In group 4, 1 mouse = positive liver, only.
[e] In group 6, 2 mice = positive liver, only.

TABLE 2. *Effects of poly A:U treatment on anti-Brucella antibodies in mouse sera sampled 53 days after immunization*

Treatment	Agglutination[a]		CF[a,b]		Hemagglutination[a]	
	−	+	−	+	−	+
None						
Controls of *B. abortus* challenge	14	0	14	0	13	1 (1:100)[c]
Saline vaccines alone						
1:10 saline vaccine	14	0	13	1 (1:40)	13	1 (1:100)
Saline vaccine	15	0	13	2 (1:40, 1:80)	13	2 (1:100)
Adjuvant vaccines alone						
1:10 H-38 vaccine	13	1 (80 IU)[d]	13	1 (1:40)	13	1 (1:100)
H-38 vaccine	11	3 (80, 320 IU)	12	2 (1:80,1:160)	12	2 (1:100) (1:200)
Poly A:U added to						
1:10 saline vaccine	14	0	12	2 (1:20)	13	1 (1:100)
Saline vaccine	14	0	14	0	14	0
1:10 H-38 vaccine	13	1 (80 IU)	13	1 (1:160)	13	1 (1:100)
H-38 vaccine	14	1 (160 IU)	14	1 (1:40)	14	1 (1:100)

[a] Figures are numbers of serologically positive (+) or negative (−) mice.
[b] CF, complement fixation tests.
[c] Figures in parentheses indicate titers of positive sera.
[d] Agglutination titers are expressed in international units (IU).

logically positive, 7 were bacteriologically positive and 4 had negative cultures.

DISCUSSION

Poly A:U added to killed vaccines of *B. melitensis* increases the immunity to infection with virulent *B. abortus* cells as evidenced by the reduction in number of *B. abortus* colonies per gram of spleen in those mice that have escaped full immunization after vaccination and poly A:U treatment. On the other hand, 300 μg of poly A:U did not enhance antibody production to the killed *Brucella* cells and soluble *Brucella* antigens that could be demonstrated as residual antibodies at the time of autopsy even though antibodies were measured by agglutination of the whole cells, by CF test, and by hemagglutination of red cells coated with a soluble antigen. This seems peculiar because poly A:U, in numerous studies by other workers, has consistently been shown to stimulate production of antibodies to a variety of antigens (1–6, 9, 11; H. G. Johnson and A. G. Johnson, Bacteriol. Proc., p. 75, 1968).

A similar situation was met in normal unvaccinated mice where chronic, as well as acute, *Brucella* infection was evidenced without clear-cut antibody production and also without gross pathologic lesions (13). In contrast, guinea pigs developed important lesions and a good circulating antibody production after a *Brucella* challenge. If there is a correlation, it exists between a serious illness and high antibody titers in most cases and in all animal species, including man, susceptible to brucellosis. The almost unique situation found in *Brucella* infection of CD-1 mice is of particular interest as a model to evaluate a possible adjuvant effect of poly A:U treatment upon cell-mediated immunity. Indeed, poly A:U has been demonstrated to stimulate thymus-derived cells of the lymphoid system (7).

Present findings would indicate that, in the mouse and *Brucella* system, 300 μg of poly A:U has an adjuvant effect on cellular immunity but does not increase production of circulating antibody. Accordingly, evidence of an adjuvant effect based solely upon the study of antibody stimulation may result in missing some agents that could be active only upon cellular immunity.

Poly A:U treatment renders more homogeneous the distribution of live *B. abortus* within mice that have escaped full immunization after vaccinations. We have no explanation for this effect which is not directly linked to immunization. It is tempting to speculate that susceptible animals are those where thymus-derived cells are insufficient in number or genetically less

active than T cells from immunizable animals. Vaccinated mice demonstrating a high degree of infection after a *B. abortus* challenge might be borderline animals that are stimulated to achieve partial immunization through poly A:U activities on T cells, as poly A:U stimulates these T cells (7). But, if challenge is near the overwhelming dose, the more susceptible animals will escape immunization, even if their immunological system is poly A:U-stimulated.

The action of poly A:U upon stimulation of immunity was assigned through regulation and activation of cyclic adenosine monophosphate (cAMP) (8). Present data may confirm an apparent parallelism between immunological events as checked by cAMP and poly A:U and known events in hormone-controlled activation of cells. These events occur as if the peak of immunization reached in mice after H-38 vaccination is unsurpassable under a stimulation by 300 μg of poly A:U. Such a situation is comparable to the effects of hormones. When injected in a giant, somatrophic hormone will not increase his weight or height. The same hormone will help stimulate subjects with potential but unused capacities.

The foregoing data must be appreciated in the light of the experimental conditions. Specific pathogen-free mice (CD-1) were employed to eliminate the remote possibility of false responses that may be due to preliminary contact of "normal" animals with specific antigenic components. A severe challenge was used (100 ID_{50}) in order to reveal any beneficial effect by poly A:U upon immunization. On the other hand, since it was possible that enhancement of immunity by poly A:U treatment might be more clearly evidenced under lesser conditions of vaccination, we also used Formalin-killed *B. melitensis* saline suspensions containing 100 or 1,000 fewer organisms than the usual H-38 vaccine. Therefore, we can conclude that 300 μg of poly A:U added to anti-*Brucella* vaccines stimulate immunization in rendering low amounts of antigens immunogenic, even against a heavy challenge.

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