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A LABORATORY REFERENCE VACCINE TO TITRATE IMMUNOGENIC ACTIVITY OF ANTIBRUCELLA VACCINES IN MICE

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Résumé

UN VACCIN ÉTALON POUR TITRER L'ACTIVITÉ IMMUNOGÉNIQUE DES VACCINS ANTI-BRUCELLA SUR LA SOURIS. — Le nombre de Brucella dans la rate de souris, 15 jours après épreuve intra-péritonéale par un inoculum standard de *Brucella abortus* dépend du statut immunitaire des animaux. Cette observation a été exploitée antérieurement pour la mise au point d'une méthode de contrôle du pouvoir immunogène des vaccins antibrucelliques. Les réponses des souris vaccinées étaient classées par rapport à des valeurs fixes, prises comme références.

Un vaccin étalon, constitué de *Brucella melitensis* souche H38 tuées par le formol puis lyophilisées, a été préparé puis titré par mesure des courbes dose vaccinale - réponse sur souris. Les doses de vaccin étalon donnant les valeurs références ont été calculées à partir de droites de régression, et exprimées en unités.

Ce vaccin rend désormais possible les comparaisons d'activités vaccinales entre laboratoires, en donnant une base d'expression de l'activité par rapport à un système unitaire.

Titration of immunogenic activity of anti-Brucella vaccines, either living or killed, must be done *in vivo* as long as mechanisms involved in anti-Brucella immunity are not better understood, by challenge of control and vaccinated laboratory animals and measure of responses. We developed, after others, a method using groups of mice vaccinated with graded doses of the vaccine to be tested, intraperitoneal challenge and enumeration of *Brucella* in spleen fifteen days after challenge (Plommet and Bosseray, 1977, 1983). This method gives reproducible and relevant results as long as strict technical points are taken into careful consideration (Bosseray *et al.*, 1983). We can, on these facts, propose a general method for the control of activity of *Brucella* vaccines (Plommet and Bosseray, 1983). However, our experience is

that, even if all technical points are well respected, some absolute differences may occur from one laboratory or one assay to the other, were it not for differences between successive groups of mice. The best way to circonvent that disagreement is to use a reference vaccine that would : 1) help standardize the technical aspects of the method, 2) check the validity of each assay, 3) give, if necessary, a standard response to one unit of the vaccine. With this purpose in mind, we previously used an oil adjuvant-killed total microbial cell vaccine (strain H38) prepared in the laboratory (Plommet and Bosseray, 1977). We chose it because we had a long experience of this type of vaccine, which has a good specific activity and is easy to prepare. Nevertheless, such a vaccine cannot be taken as a laboratory reference, since : 1) oil adjuvant is impossible to standardize exactly, 2) stability of emulsion cannot be permanently assured, 3) dilution of the bacteria suspension in oil may introduce inaccuracy. To avoid these drawbacks, we have tried several substitutes : a formalin-killed cell suspension of *Brucella* strain H38 lyophilized in a polyvinylpyrrolidone solution was chosen as the best reference vaccine. We describe here preparation and titration of this vaccine.

Materials and Methods

Preparation of the reference vaccine

The strain *Brucella melitensis* 53 H38 (H38) was originally given by G. Renoux and kept lyophilized, as indicated below, for standard challenge. The lyophilized strain was first suspended in water and seeded on TSA to test, after incubation, the smooth phase of individual colonies under oblique reflected light, by direct examination and after staining, as indicated by Alton *et al.* (1975). (A strain having more than 5 % rough colonies must be discarded.) Otherwise the strain has to be purified so that only smooth bacteria would be harvested). The strain was then cultivated on TSA in Roux flasks at 37 °C for 40 h. Bacterial cells were harvested in Buffer saline solution (BSS : NaCl, 9 g ; H_2HPO_4, 2 g ; KH_2PO_4, 1 g ; distilled water, 1 000 ml ; pH 6.85), washed once, centrifuged, then killed in BSS-added formalin 1 % (w/v) and left at 37 °C for 24 h then at 20 °C for three days. The cells were washed again by centrifugation, and suspended in BSS plus formalin 0.1 % so that there were about 10^{12} cells per ml. Checking of sterility was done on washed suspension, diluted to about 10^8 Brucella/ml, seeded on TSA Petri plates and in Trypticase Soy Broth and incubated for at least eight days.

This is the « fresh » suspension used in this paper (table 1). It keeps its immunogenicity for at least three years stored at 4 °C. It can be used as a « working » reference vaccine.

Lyophilized reference vaccine, used in this paper was prepared as follows : The freshly killed 10^{12} *Brucella* suspension was washed again, and suspended in a solution of polyvinylpyrrolidone (pvp 1 %, filtrated on 0.22 µm millipore membrane). Bacterial concentration was then checked by nephelometry (Beckman B, 600 nm) and diluted to the required concentration. We prepared two batches : no. 1 contained 4.1×10^{10} cells per ml and no. 2, 1.2×10^{11} and we pipetted respectively 0.75 ml and 0.40 ml per 5 ml ampoule. These doses were chosen so that at least 30 mice could be vaccinated at 15 units with one ampoule. Lyophilization was performed as indicated below, for standard challenge. Ampoules were sealed under vacuum.

Preparation of the standard challenge

The strain *B. abortus* 544, originally obtained from the Central Veterinary Laboratory, Weybridge, was used as challenge, in lyophilized state. It was diluted to the standard dose of 2×10^5 living bacteria just before challenge. This standard challenge was prepared as follows :

The strain was first checked for the smooth phase, then seeded onto TSA medium in Roux flasks and incubated at 37 °C in 10 % CO_2 for 30 h. The strain was harvested in BSS, washed, titrated by nephelemetry and diluted to about 7.5×10^{10} bacteria per ml in the following solution for lyophilization (Greaves, 1962) : bovine albumine, 5 g ; saccharose, 7.5 g ; sodium glutamate, 1 g ; distilled water, 100 ml ; sterilization by filtration on Millipore membrane 0.22 µm. The suspension was distributed (0.2 ml) in 5 ml sterile ampoules and lyophilized with a Speedivac Edwards 30 PI/637 apparatus by a three step process : freezing by centrifugation under vacuum, 1 h ; main dessication, 20-22 h ; last dessication on manifold before sealing ampoules under vacuum, 5 h. About 70 % of bacteria would survive the process (about 1×10^{10} per ampoule). The number of living bacteria and virulence of the strain have then to be checked carefully. They remained unchanged for at least five years when stored at 4 °C.

Control of viability was done on at least three ampoules. Each sample was suspended in 1 ml

Table 1. — Comparative titration of four samples of reference vaccine, batch no. 1. Dilutions of fresh or reference lyophilized were administered to lots of six CD-1 mice. Doses, roughly estimated in previous assays, were expressed in units. Fresh suspension (not lyophilized) was used at the same dose (number of bacterial cells) as lyophilized one. Challenge : 1.88×10^5 *Brucella*

	Dose (Unit/mouse)				
	Estimated 0 Exact[b] 0	0.25 0.29	1 1.18	4 4.72	16 18.86
Vaccine					
Fresh suspension	4.88 ± 0.22[a]	3.06 ± 0.66	2.95 ± 0.86	2.57 ± 0.39	2.60 ± 0.28
Lyophilized ampoule					
1	4.77 ± 0.43	3.41 ± 0.88	2.56 ± 0.44	2.73 ± 0.68	2.13 ± 0.36
2	4.89 ± 0.40	2.85 ± 0.86	2.59 ± 0.72	2.45 ± 0.63	1.85 ± 0.75
3	4.98 ± 0.38	3.06 ± 0.86	2.82 ± 0.72	2.63 ± 0.32	2.18 ± 0.27
Total mean response ± rsd	4.88 ± 0.37	3.10 ± 0.82	2.73 ± 0.70	2.60 ± 0.53	2.19 ± 0.46

Overall residual standard deviation : 0.60.
a : Mean ± standard deviation.
b : titer calculated from regression of response to dose (0.25 to 4 estimated Units).
$Y = 2.81 - 0.42$ log (estimated units) 1 Unit (response = 2.84) = 0.85 estimated Units.

of SST and diluted to 1×10^{-4} : 0.2 ml of this suspension would contain about 2×10^5 bacteria, the correct challenge dose. This suspension was diluted again to 1×10^{-3} and 0.2 ml seeded onto at least six TSA plates. After incubation, the exact number of viable bacteria was determined (and appropriate dilution for challenge modified if required). Exact enumeration of challenge was done routinely at each assay, on at least six plates.

When preparing a new batch of standard challenge ampoules, virulence of the strain must be checked once (after lyophilization as previously indicated) (Plommet and Bosseray, 1977). Five to eight week old CD-1 mice must be intraperitonally challenged with three doses : 5×10^2, 1×10^4 and 2×10^5 (six mice per dose). The responses should fit those given previously that is about 2.8 to 3 for the lowest doses, and 4.78 (limit : 4.47) for the standard dose.

Mice and experimental design

Groups of six female, six week old CD-1 mice (Charles River, Elbeuf, France) bred under our standard controlled conditions (Plommet and Bosseray, 1977) received subcutaneously 0.1 ml of the appropriate dilution of reference vaccine in BSS, either fresh or lyophilized rehydrated. The dilutions used were chosen so that each mouse received graded doses below and above one roughly estimated unit (table 1 and 2). The rough estimation was done by a preliminary assay. Control groups received BSS alone.

Thirty days after vaccination, the mice were challenged intraperitoneally, after a 16 h starvation to make sure that the challenge was really administered into the peritoneal cavity. The dose injected was about 2×10^5 bacteria, in 0.2 ml BSS. The exact number was determined for each assay.

Fifteen days after challenge, the mice were killed, for enumeration of *Brucella* in spleen, as previously described (Plommet and Bosseray, 1977). X being the number of colony forming unit (CFU) per spleen (when no colony was found, we considered the spleen infected by five bacteria), the results were expressed by Y,

$$Y = \log(X/\log X),$$

so that distribution of Y would follow conditions for analysis of variance (Bosseray and Plommet, 1976). Mean and standard deviation of Y are the responses of the groups of six mice.

Results

Two batches of reference lyophilized vaccines were prepared. The " fresh " suspension (before lyophilization) was tested comparati-

Table 2. — Comparative titration of three samples of reference vaccine, batch no. 2. Challange : 2.07×10^5 *Brucella*. See legend table 1

	Dose (Unit/mouse)					
	Estimated 0 Exact[b] 0	0.04 0.06	0.20 0.31	1 1.53	5 7.67	25 38.3
Lyophilized ampoule						
1	4.91 ± 0.30	3.49 ± 0.66[a]	2.75 ± 1.09	2.76 ± 0.61	1.95 ± 0.98	2.44 ± 0.45
2	4.98 ± 0.19	4.16 ± 0.69	3.33 ± 0.93	3.23 ± 0.75	2.25 ± 0.43	1.85 ± 0.68
3	4.70 ± 0.46	4.22 ± 0.53	3.22 ± 0.70	2.76 ± 0.34	2.18 ± 0.85	2.12 ± 0.69
Total mean response ± rsd	4.87 ± 0.33	3.96 ± 0.64	3.10 ± 0.92	2.91 ± 0.59	2.12 ± 0.79	2.14 ± 0.62

Overal residual standard deviation = 0.67.
a : Mean ± standard deviation.
b : titer calculated from regression of response to dose (0.20 to 5 estimated Units).
$Y = 2.71 - 0.70 \log$ (estimated Unit). 1 Unit (response = 2.84) = 0.65 estimated Units.

vely to dilutions of three different ampoules of batch 1 in the first assay (table 1). Three ampoules of the second batch were compared in the second assay (table 2).

Before analyzing responses to the vaccine tested, we should first test conformity of responses to the model (Plommet and Bosseray, 1977) : mean responses of control groups were respectively 4.88 and 4.87, values very close to those obtained before, that was 4.72 (Plommet and Bosseray, 1977) and obtained since that on 20 control groups, that is 4.78 (Bosseray *et al.*, 1983). Residual standard deviations were respectively 0.60 and 0.67, very close again to our previously published (0.59) and new data (in all 176 groups), 0.59.

Killed vaccines induced a dose-dependent response characterized by a maximum response zone : mean response of the best immunizing vaccines (2.18) plus one least significant difference between two lots of six mice (lsd = 0.06 for P = 0.05) (Bosseray *et al.*, 1983). The optimal dose (OD) (Plommet and Bosseray, 1977) was the vaccine dose giving a response at the limit of maximum zone. We now introduce the vaccinal Unit (U) that is the activity of 1 OD (response 2.84).

The responses to four doses of four samples of batch 1 vaccine were given in table 1. Analysis of variance indicated that no differences were recorded between samples (F = 1.24). The dose-response curve was analysed as a linear regression. This regression was indeed approximately linear between 0.25 and 4 Units. The exact titer of the vaccine was then computed from it, taking the reference value 2.84 as the response to 1 Unit. On this basis, the estimated 1 Unit dilution contained in fact 1.18 U. And each ampoule of batch 1 reference vaccine contained 547 Units.

The responses to 5 doses of the three samples of batch 2 vaccine are given in table 2. Again no difference was shown between samples (F = 1.25). From the linear regression, the exact titer of estimated U dilution was calculated : it was 1.53 U, and the total amount per ampoule was 1 100 U. We consider, from now on this batch as the reference vaccine.

Discussion

Because the number of CFU of *Brucella* in the spleen of mice fifteen days after a standard (strain and dose) intraperitoneal challenge is a good measure of anti-Brucella immunity, we developed a control method that compared mean responses of mice to a reference scale. This included average responses of control (non-vaccinated) mice, average responses to vaccines known from field experiments to be good, and pooled standard deviation of responses (Plommet and Bosseray, 1977). In routine controls of activity, only a few groups of mice can be used. Hence, criteria for acceptance or refusal of batches of vaccines should consider the statistical fluctuations of responses. This point is discussed elsewhere (Bosseray *et al.*,

1983). We had, however, previously taken this into consideration when we proposed that a good vaccine should give a response equal to or below 2.84, the value which was defined as the observed mean response to good vaccines (suspended in oil adjuvant) (2.18), plus one least significative difference (lsd) between two groups of six mice (0.66), calculated from the pooled standard deviation. This proposal made the value 2.84 the main basis of the method. We had defined the theoretical vaccinal dose giving this response as the optimal dose (OD). Having gathered many more data that convinced us of the repeatability of responses, we now feel it more advisable to reverse the proposal, and consider that the reference value is that given by *one Unit* of a reference vaccine. We chose to define the *Unit* as the activity of one OD, which in our hands gives the response 2.84.

Hence we had to make a reference vaccine, the titer of which was to be expressed in Units. Such a vaccine must, 1) be easy to prepare, to store and to exchange between laboratories, 2) be stable on conservation, 3) be easy to use, particularly to dilute in saline solution. These requirements exclused the oil adjuvant vaccines we used previously. Since in mice simple formalin-killed suspension of appropriate *Brucella* strains are highly immunogenic, we chose to lyophilize such a suspension of strain *B. melitensis* H38 in pvp solution. Our results showed that responses to the new reference vaccine we prepared were very similar to those we obtained previously. In particular, the standard deviations were approximately equal and the mean responses were well related to doses. We could easily, with this vaccine, determine the references values : not only the limit response to 1 Unit, but also, for example, the mean response to good vaccine, showed equal to 2.18 (Plommet and Bosseray, 1977 ; Bosseray *et al.*, 1983). From the common regression equation based on pooled data from tables 1 and 2, which would give the estimation of the dose-response curve between 0.29 and 7.67 Units, ($Y = 2.84 - 0.56 \log U$), the value 2.18 was obtained at a vaccinal dose of 15 Units. These 1 U and 15 U points define precisely the zone of response including all good vaccines (maximum response zone).

Moreover, we used this reference vaccine to compare responses of different breeds of mice (Bosseray *et al.*, 1983). It could be used the opposite way to define reference values if CD-1 strain of mice was not available and another breed had to be used.

The two batches of reference vaccine we prepared had approximately the same activity : the number of bacterial cells per Unit were respectively 5.6 and 4.4×10^7. This number is about twice as big as the one obtained if the vaccine were added oil adjuvant after appropriate dilution. But because oil adjuvants cannot be conveniently standardized between laboratories and stored, we introduce this laboratory-lyophilized vaccine as a reference, which keeps its activity — as far as we know — for at least three years and very likely for many more years.

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Summary

The number of bacteria (CFU) in spleens of mice fifteen days after a standard intraperitoneal challenge of *Brucella abortus,* is dependent on vaccinal immune status of mice. From this observation, a control method of vaccinal activity was previously proposed, which classified the responses in relation to fixed reference values. A reference vaccine, a lyophilized formalin-killed bacterial cell suspension of *B. melitensis* strains H38 was prepared and titrated. From the dose response curve, the quantities giving reference values were calculated and expressed in a Unit system. This vaccine makes possible inter-laboratory comparisons of vaccinal activity, that can be expressed on the Units basis.

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