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RELATION BETWEEN MACROPHAGE MIGRATION INHIBITION AND IMMUNITY TO *BRUCELLA ABORTUS* IN GUINEA-PIGS

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ABSTRACT

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A soluble protein antigen was prepared from *Brucella melitensis* Rev I with which macrophage migration inhibition (MMI) assays were successfully done using guinea-pig peritoneal exudate cells.

By comparing the MMI, agglutinin response and immunity of groups and of individual guinea-pigs which had been immunized with either *B. melitensis* Rev I live vaccine or *B. melitensis* Rev I inactivated antigen, an association between the MMI and resistance to infection was demonstrated.

Résumé

Le rapport entre le test d'inhibition de migration de macrophages et l'immunité à Brucella abortus *chez le cobaye.*

Em employant un antigéne protèique soluble préparé à partir de Brucella melitensis *Rev I, les auteurs ont pu réaliser avec succès le test d'inhibition de migration de macrophages (IMM) avec cellules exsudatives provenantes du péritoine du cobaye.*

Une comparaison de l'IMM, la réponse immunitaire en agglutinines et l'immunité de cobayes en groupes et individuellement qui ont étés immunisés soit avec un vaccin vivant de B. melitensis *Rev I soit avec un antigène inactivé de* B. melitensis *Rev I, a permise les auteurs à constater un rapport entre l'IMM et la résistance des cobayes a une infection expérimentale.*

INTRODUCTION

The mechanism through which immunity to brucellosis is established in immunised animals is unknown. *Brucella* organisms are intracellular parasites and thus have much in common with parasites such as *Mycobacterium tuberculosis* and *Listeria monocytogenes* in which cellular immunity is a prominent feature (Mackaness, 1962; Mackaness, 1964; Hahn, 1974). Moreover, it is common knowledge that cattle that have been immunized between the ages of 3–4 months are still protected against infection long after serum antibody levels become undetectable by conventional serological tests (Gregory, 1958). It is reasonable to expect, therefore, that cellular immune mechanisms will also feature prominently in the protection against brucellosis. In fact it has been shown that macrophages from immunized rabbits exhibit an enhanced though transient capacity to inactivate brucellae (Ralston & Elberg, 1968a; Ralston & Elberg, 1969; Ralston & Elberg, 1971a).

Conversely, it has been found that specific antibodies enhance the bactericidal capacity of macrophages (Ralston & Elberg, 1971b; Ralston & Elberg, 1971c), and it has been suggested that these may either be cytophilic (Ralston & Elberg, 1968b) or may be incomplete antibodies which are not readily detectable (Beh, 1975).

The inhibition of macrophage migration is a well-established correlate of delayed type hypersensitivity (DTH) (Melnick, 1971; Curtis & Hersh, 1973), but the situation regarding the relation between DTH, inhibition of macrophage migration and immunity is not clear. Jones & Berman (1971) found that in guinea-pigs long-lasting immunity to *Brucella* infections was conferred only by vaccines that induced DTH and were primed for antibody production, but they were unable to dissociate these effects. Simon & Sheagren (1972) were unable to establish any correlation between the production of macrophage inhibition factor (MIF) and enhanced ability of macrophages to destroy *L. monocytogenes*. It should nevertheless be remembered that, as in the case of tuberculosis and salmonellosis, immunity may also be dependent on the conjoint action of immune cells and immune serum (Vickrey & Elberg, 1971; Cameron & Van Rensburg, 1975).

Since no correlation has been demonstrated between conventional serum antibodies and immunity to brucellosis, we proposed to determine whether a correlation exists between inhibition of macrophage migration and protection against infection with *B. abortus*. Horwell & Van Drimmelen (1972) showed that *B. melitensis* Rev I induces a solid immunity in cattle against infection with *B. abortus* and this prompted the use of this system as a safety measure in guinea-pigs in this study.

The antigen used in the migration inhibition procedure is naturally important and should ideally be the component of the organism that is responsible for the inducement of immunity. Crude cell wall preparations of brucellae have been found to be immunogenic (Keppie, Witt & Smith, 1963), and sonic extracts have been used successfully in macrophage migration inhibition studies in mice (Sandok, Hinsdill & Albrecht, 1971). Chen & Elberg (1969) were able to induce a good immunity in guinea-pigs with an antigen prepared by ammonium sulphate precipitation. Later Ralston & Elberg (1971a) showed that the capacity of the Rev I strain of *Brucella melitensis* to prime stem cells was associated with a particular protein antigen. More recently, Jones, Diaz & Taylor (1973) found that a protein antigen was instrumental in inducing DTH in infected guinea-pigs. Subsequently Jones & Berman (1975) established that DTH reactions, uncomplicated by accompanying antibody-mediated reactions, were seen only in infected guinea-pigs with protein antigen that was entirely free of lipopolysaccharide.

Thus, as both immunity and DTH appear to be mediated by protein antigens, it was decided to use protein antigens in the macrophage migration inhibition assays as well.

MATERIALS AND METHODS

Bacterial strains

B. melitensis Rev I was used for preparing the live vaccine, the antigen for agglutination tests and crude soluble protein antigens employed in the macrophage migration inhibition tests. *B. abortus*

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strain 544 (Compton) was used to challenge immunized guinea-pigs. Both cultures were maintained in the lyophilized state.

Experimental animals

Conventionally-reared, random-bred guinea-pigs obtained from the colony maintained at the Institute were used throughout. They were fed a pelleted ration supplemented with fresh lucerne. Unless stated otherwise, all guinea-pigs were 4–6 months old.

Vaccines

Lyophilized *B. melitensis* Rev I vaccine was obtained from the vaccine production section of the Institute. The reconstituted vaccine contained a minimum of 1×10^9 viable organisms/ml.

The *B. abortus* 45/20 oil emulsion vaccine (Duphavac N.A.) was kindly supplied by Philips-Duphar B.V., Amsterdam, Holland.

Serological tests

Antigen for assaying the agglutinin response in guinea-pigs was prepared from *B. melitensis* Rev I as described by Alton & Jones (1967) for the preparation of *B. abortus* antigen. This antigen was also used to immunize guinea-pigs in certain experiments, the tests being conducted and interpreted according to Alton & Jones (1967). Serum collected from the guinea-pigs killed for MMI tests was used for assaying the agglutinin response.

Macrophage migration inhibition tests

Mass cultivation of *B. melitensis* Rev I for preparing soluble antigens was done by the method described by Van Drimmelen (1956) for *B. abortus* S19.

Two soluble protein antigens were employed, one of which was a cold NaCl extract prepared by the method of Jones, Diaz & Taylor (1973), and the other a veronal buffer extract (Barber, Eylan & Keydar, 1968; Barber & Eylan, 1972) prepared by the method of Cameron & Van Rensburg (1975), except that the first step employing alcohol treatment was omitted. The tests themselves were also done as described by Cameron & Van Rensburg (1975), except that foetal calf serum was used for the cell cultures instead of guinea-pig serum. The same formula for calculating the percentage inhibition of migration was used.

Immunization and challenge of guinea-pigs

Guinea-pigs were immunized with either live Rev I vaccine, Duphavac or Rev I antigen. The Rev I live vaccine and Duphavac were administered intramuscularly and the Rev I antigen intraperitoneally, all 3 vaccines being administered in 1 ml dosages.

Unless otherwise indicated, guinea-pigs immunized with Rev I vaccine or antigen were challenged 6 weeks after immunization by the intramuscular injection of 5×10^3 virulent *B. abortus* strain 544 (Compton) organisms. Guinea-pigs which received Rev I antigen were normally challenged 2 weeks after immunization. In all instances, experiments were initiated with more animals than were actually required in order to compensate for individuals which did not yield satisfactory peritoneal exudate cells.

Two sets of non-immunized control groups were included in all the immunity experiments. Of these the positive controls were challenged, but not the negative ones.

All the challenged guinea-pigs, including both the positive and the negative controls, were killed 6 weeks after exposure and their individual spleen masses determined. This is an established method for assaying the potency of *Brucella* vaccines (Todd, 1970).

Experiments

Toxicity of soluble B. melitensis Rev I antigens. Peritoneal exudate cells (PEC) were collected from 2 normal guinea-pigs and cultures were prepared in the presence of 0; 5; 10; 25 and 50 μg/ml of NaCl extract and veronal extract antigen, respectively.

Optimum concentration of antigens. PEC were collected from 3 guinea-pigs which had been immunized with Rev I vaccine 6 weeks previously and the percentage macrophage migration inhibition (MMI) was determined in the presence of 12,5 and 25 μg/ml NaCl and veronal antigens.

Effect of pre-incubation with veronal antigen on MMI. Since it has been claimed that pre-incubation of cells with antigen promotes MMI (Gárski, Orlowski, Pomorski & Kwiek, 1973; Philip, Johnson & Spencer, 1973), the effect of this manipulation on the system was also tested. PEC were collected from 3 immunized guinea-pigs and aliquots of each pre-incubated with 25 μg/ml veronal antigen for 0; 3; 6 and 24 h, respectively, before preparing the cultures.

Duration of agglutinin response and MMI. A group of 72 6-month-old guinea-pigs was immunized with *B. melitensis* Rev I live vaccine. Serum and PEC were collected from 9 animals as well as from 9 non-immunized controls after 1 week, 2 weeks and then after 1, 2, 3, 4, 5 and 6 months. The agglutinin titre and MMI of 8 of the guinea-pigs were determined at each interval and the mean values plotted.

Comparison of MMI, agglutinin response and immunity 6 months after immunization. A group of 16 6-month-old guinea-pigs was immunized with *B. melitensis* Rev I live vaccine.

After 6 months, the MMI and agglutinin responses of 6 of these guinea-pigs were determined. Six others, as well as controls, were challenged with virulent *B. abortus* and 6 weeks later their spleen mass was determined as a criterion for resistance to infection.

Another group of 16 animals was immunized with *B. melitensis* Rev I antigen $5\frac{1}{2}$ months after the beginning of the experiment and the MMI, agglutinin response and immunity assayed 2 weeks later, as outlined above.

Effect of age on the immune response. Three groups of 56 guinea-pigs aged 4 weeks, 3 months and 6 months, respectively, were used. The animals in each age group were treated as follows: 16 were immunized initially with *B. melitensis* Rev I live vaccine and 2 weeks later another 16 were immunized with *B. melitensis* Rev I antigen. Four weeks after the beginning of the experiment animals from each of these groups were challenged and 8 were used to determine the agglutinin response as well as the percentage MMI. The 24 non-immunized animals served as controls for the MMI and immunity assays.

Immune response to Duphavac. Two groups of 15 guinea-pigs were used in the following experiment, performed in the anticipation that Duphavac may render guinea-pigs immune in the absence of a serum antibody response (Roerink, 1966). The 1st group was given 2 intramuscular injections of 1 ml Duphavac at an interval of 6 weeks and their agglutinin response, percentage MMI and immunity

were assayed 5 weeks after the 2nd injection, as in the previous experiment. The 2nd group was given *B. melitensis* Rev I vaccine followed 4 weeks later by Duphavac and they, as well as controls, were assayed 2 weeks after the last injection.

Comparison of spleen masses and percentage MMI of individual guinea-pigs. A group of 24 3–4-month-old guinea-pigs was immunized with *B. melitensis* Rev I live vaccine. They, as well as 24 non-immunized controls, were assayed 4 weeks later. PEC and serum were collected from these guinea-pigs without killing them and the same animals were challenged with virulent *B. abortus* a week later. Numerous animals succumbed during this procedure, but the percentage MMI of the survivors was plotted against their individual spleen masses.

RESULTS

Toxicity and efficacy of antigens

As shown in Table 1, even a concentration of 50 μg/ml of either the veronal or NaCl soluble antigens exhibited minimal toxicity on the PEC and did not inhibit their migration from the capillary tubes.

TABLE 1 Effect of various antigen concentrations on migration of normal PEC

Antigen concentration μg/ml	Area of migration Arbitrary units			
	Veronal antigen		NaCl antigen	
	GP 1	GP 2	GP 1	GP 2
0	18,04	38,52	18,04	38,52
5	18,67	39,89	18,67	28,29
10	22,72	37,14	24,86	33,19
25	23,77	35,80	19,64	39,86
50	27,77	31,93	21,66	38,52

There was, however, an appreciable difference in the efficacy with which the 2 antigens were able to elicit MMI (Fig. 1). The veronal antigen was distinctly superior to the NaCl, and 25 μg/ml gave excellent results, but to obviate inaccuracies due to mass-measuring faults, a concentration of 50 μg/ml antigen was used in the subsequent experiments. A typical result obtained by using 50 μg/ml antigen, showing distinct MMI in the presence of antigen as opposed to a control without antigen, is shown in Fig. 2.

In our hands pre-incubation of PEC with antigen was not successful (Fig. 3). In 2 of the 3 guinea-pigs tested, pre-incubation for 6 h and 24 h had a detrimental effect on MMI, but in the other instance this was not the case. Tests done with cells immediately after collection gave consistently good results.

Duration of the immune response

The agglutinin response in *B. melitensis* Rev I-immunized guinea-pigs rose to a peak within 2 weeks and then dropped again to a low level within 3 months (Fig. 4). Despite this initial rapid fall some animals were still distinctly positive 6 months after immunization.

The MMI response rose more slowly, reaching a peak after 1 month and gradually declining to a plateau after 6 months. This persisted until the end of the experimental period.

As Fig. 5 shows, guinea-pigs challenged 6 months after immunization with *B. melitensis* Rev I live vaccine were all solidly immune and exhibited a fair level of MMI but they also had appreciable agglutinin titres. In contrast, guinea-pigs which were challenged 2 weeks after immunization with *B. melitensis* Rev I antigen showed poor MMI, generally had high agglutinin titres and were appreciably less resistant to infection than the 1st group. The results in Fig. 5 thus clearly indicate that there is a relation between MMI and protection against infection but not between antibody levels and immunity.

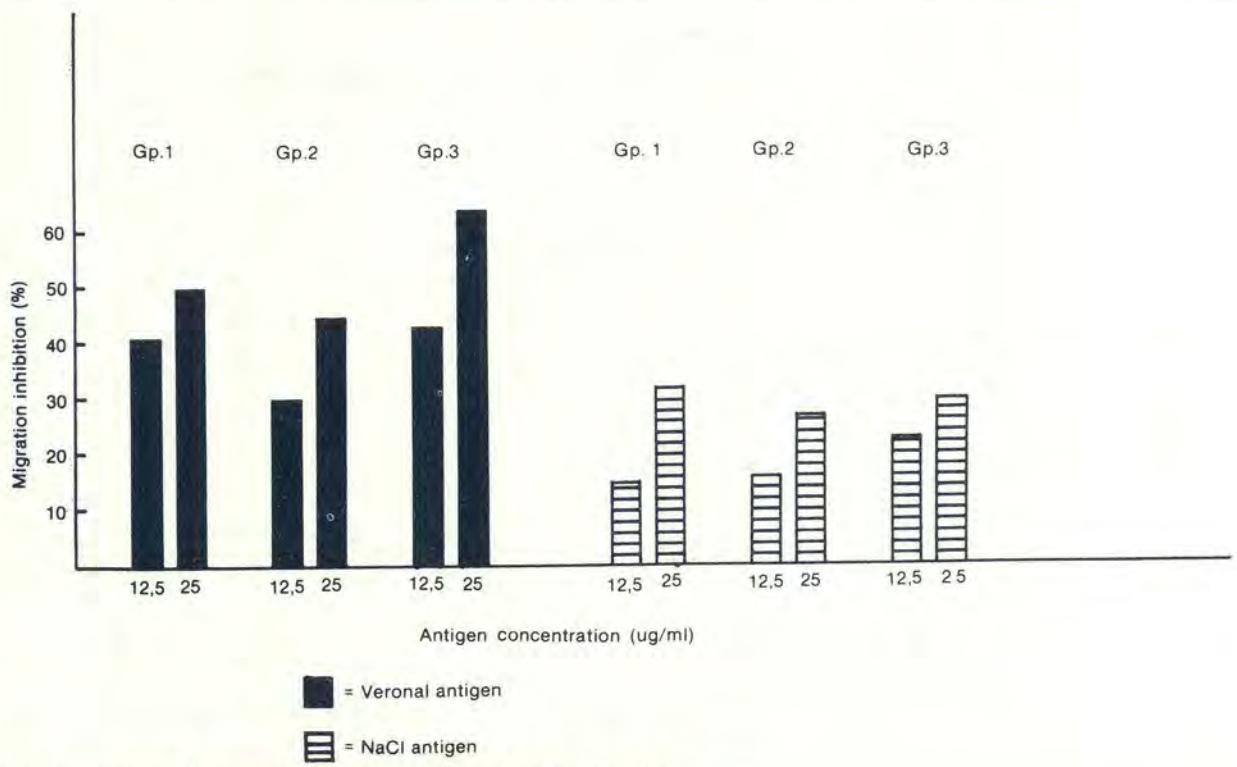

FIG. 1 Comparison of migration inhibition by veronal and NaCl antigens

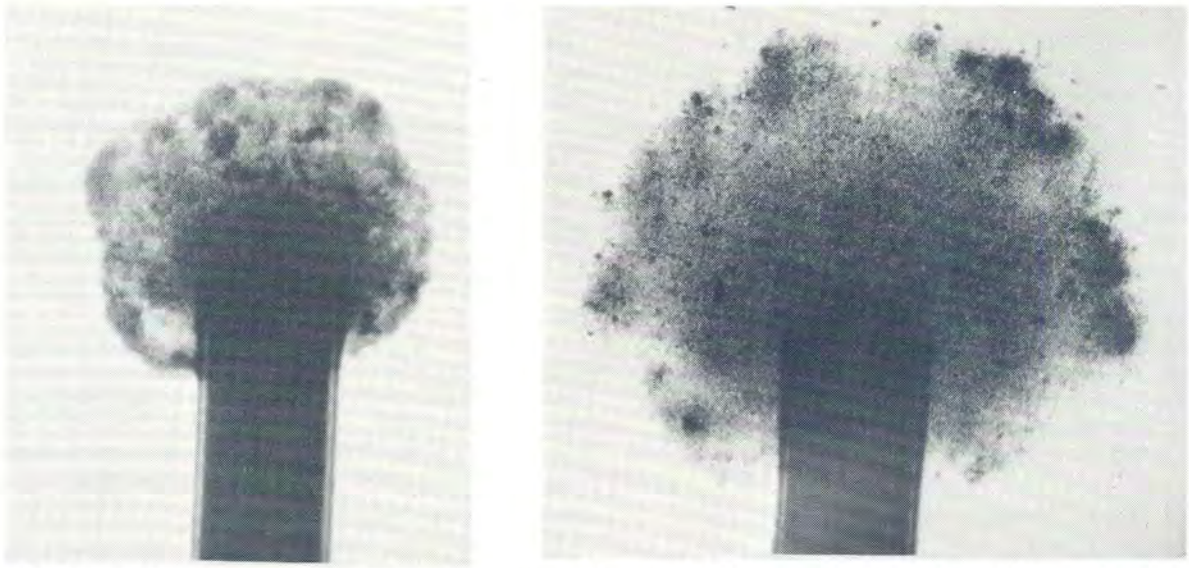

FIG. 2 Inhibition of macrophage migration in the presence of antigen (left) compared with a culture without antigen (right)

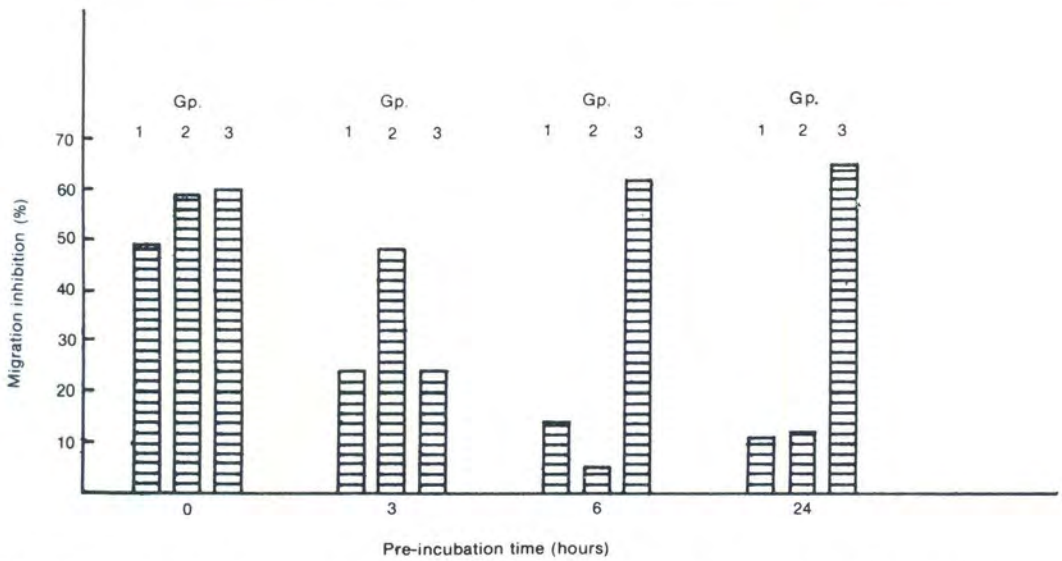

FIG. 3 Effect of pre-incubation of peritoneal exudate cells with veronal antigen on migration inhibition

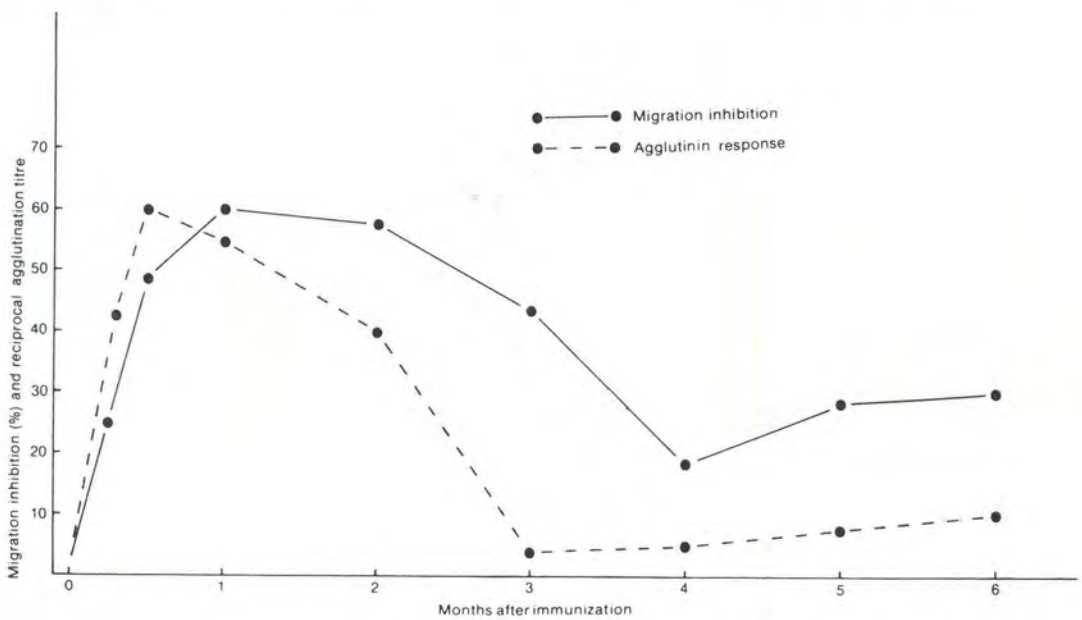

FIG. 4 Duration of agglutinin response and macrophage migration inhibition in *B. melitensis* Rev I immunized guinea-pigs

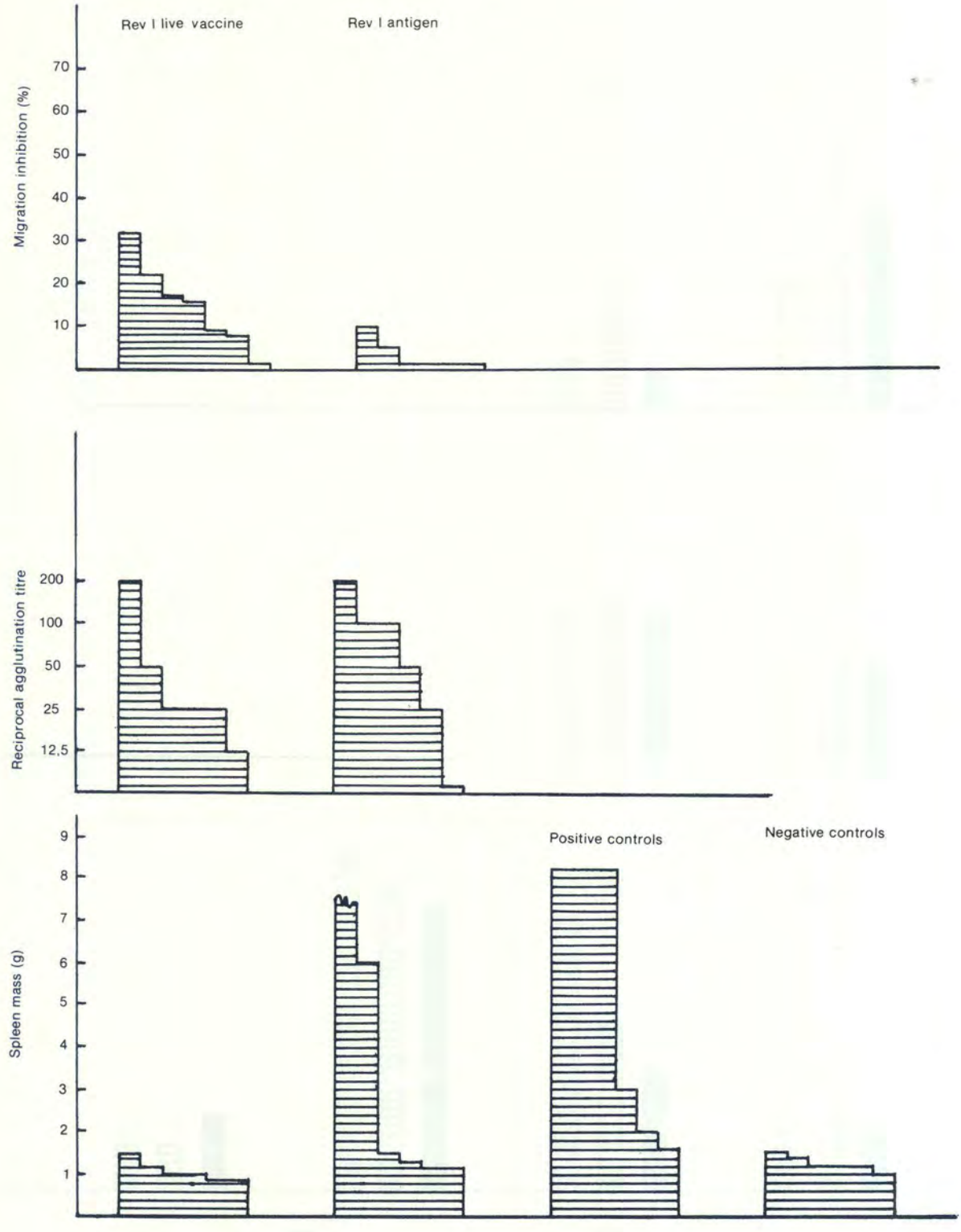

FIG. 5 Macrophage migration inhibition, agglutinin titre and spleen mass of guinea-pigs challenged 6 months (Rev I vaccine) and 2 weeks (Rev I antigen) after immunization

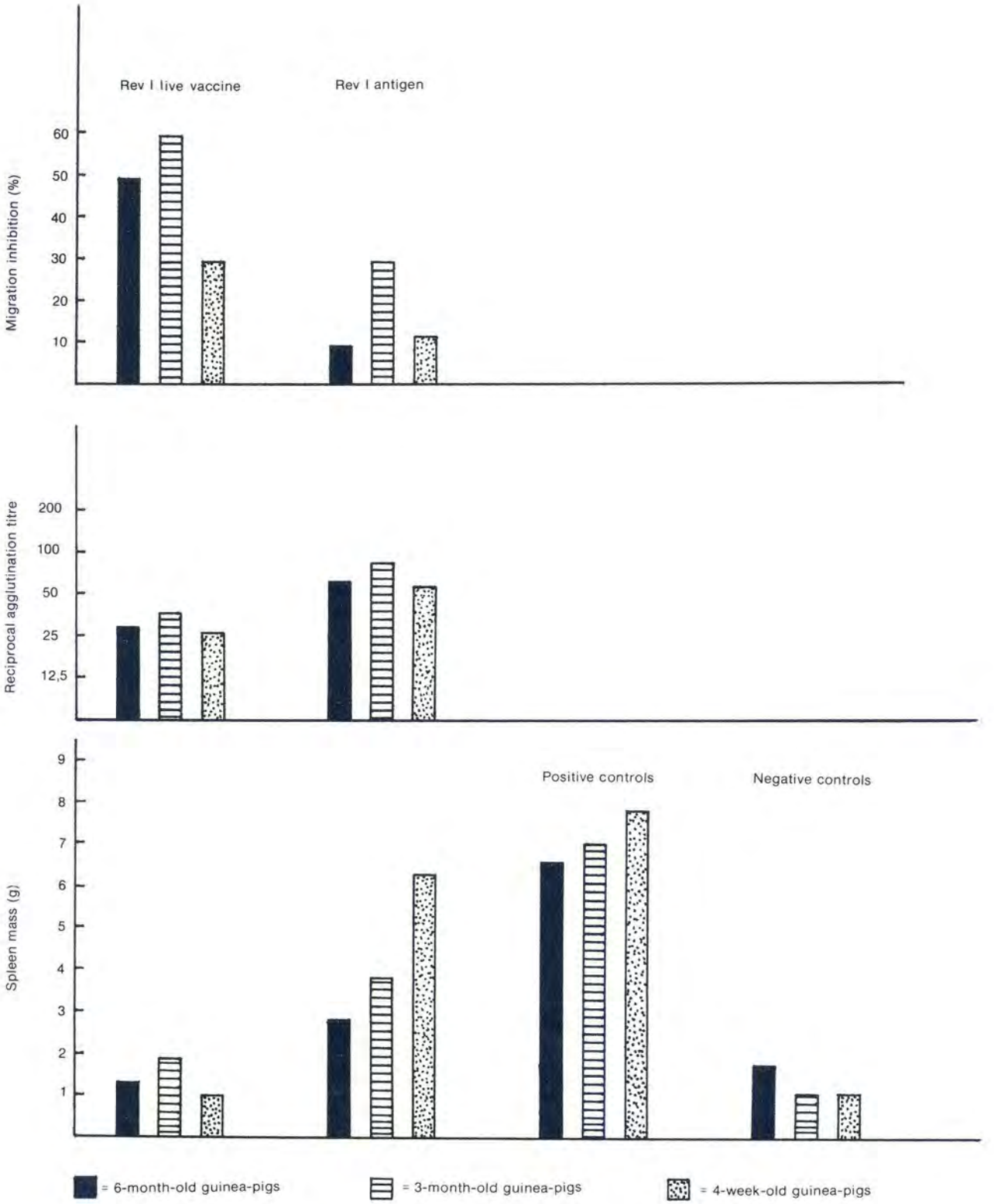

FIG. 6 Macrophage migration inhibition, agglutinin response and spleen mass of guinea-pigs immunized at different ages

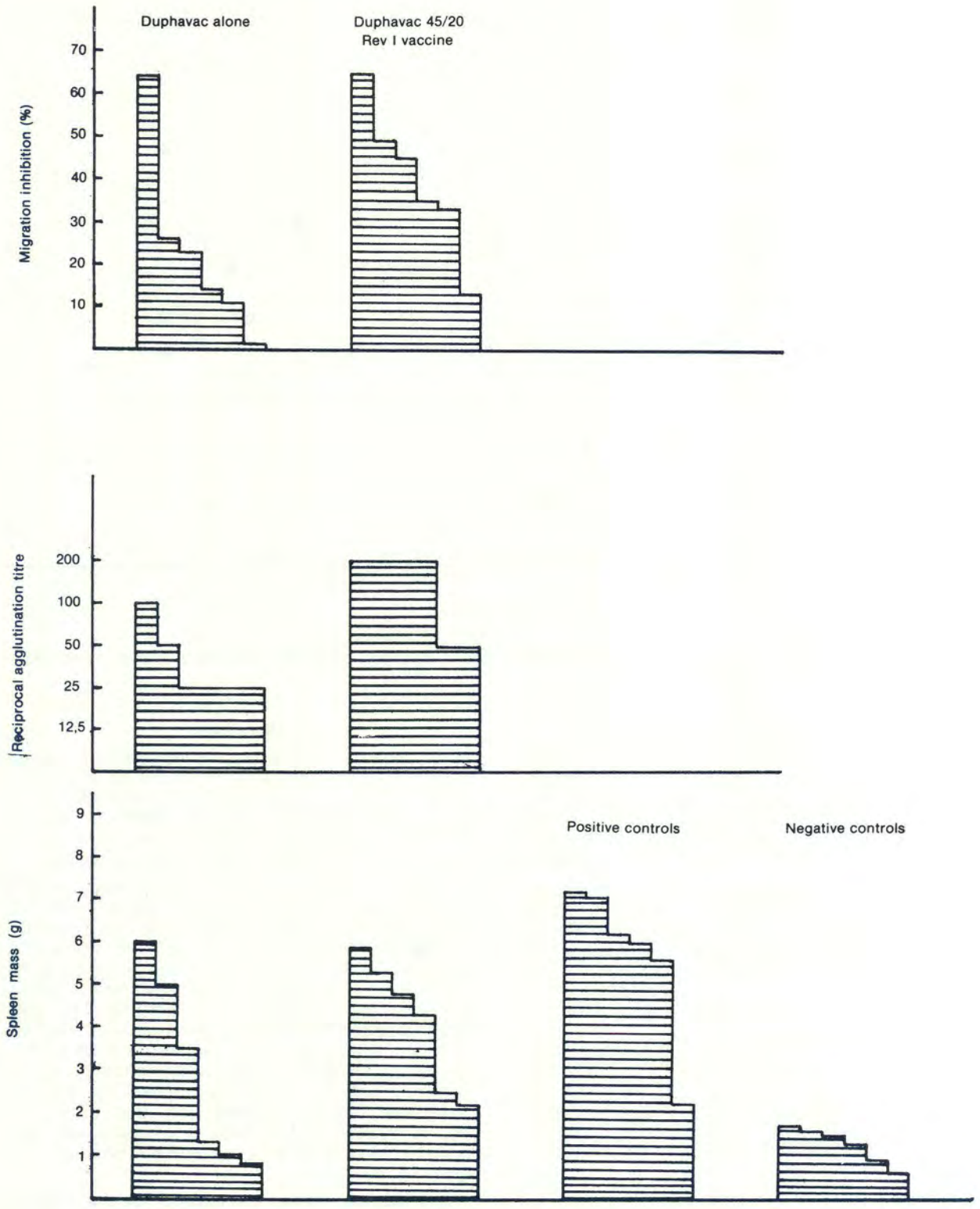

FIG. 7 Macrophage migration inhibition, agglutinin response and spleen mass of guinea-pigs immunized with Duphavac 45/20 alone and Duphavac 45/20 plus Rev I vaccines

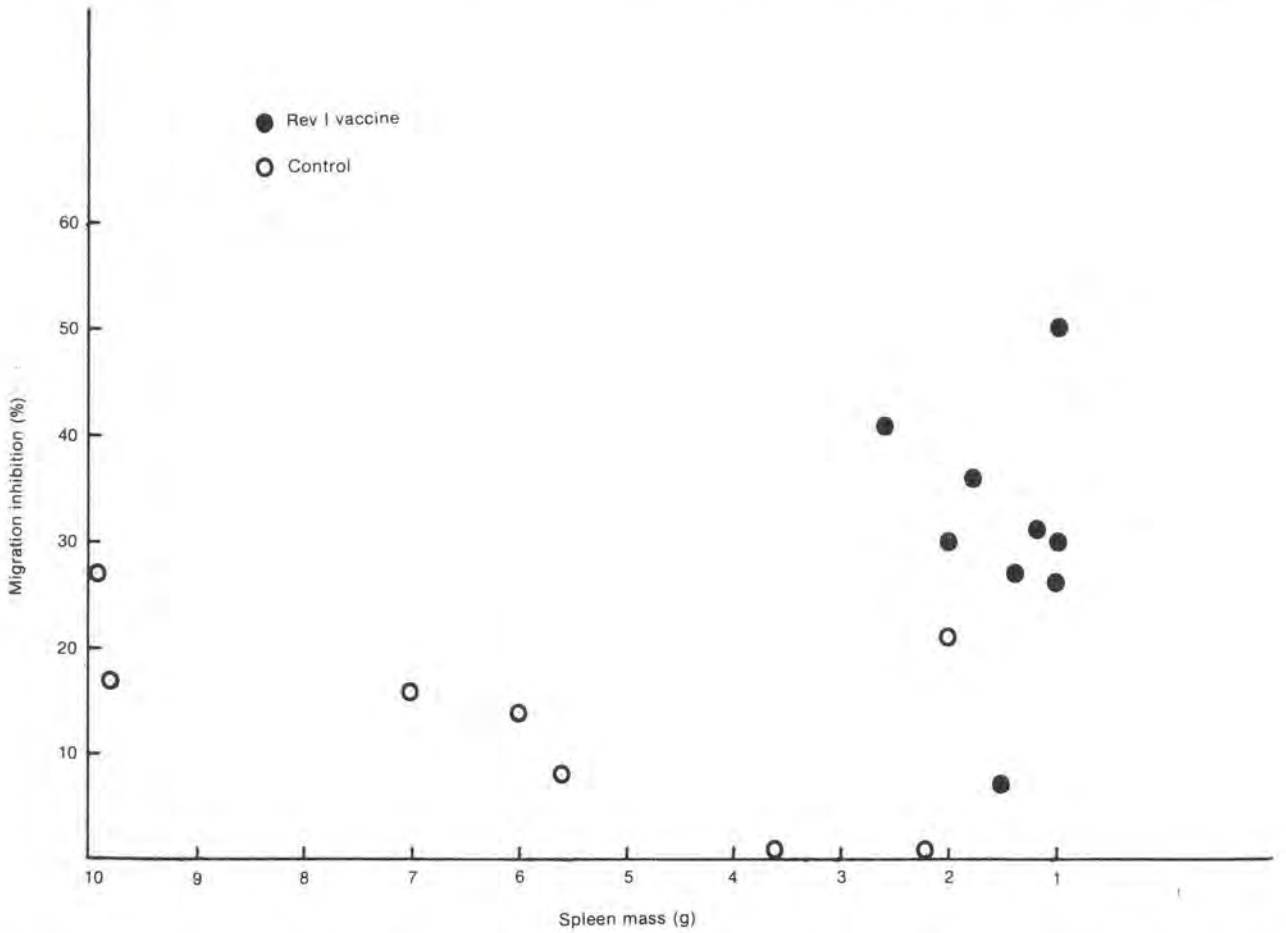

FIG. 8 Macrophage migration inhibition compared with spleen mass of individual guinea-pigs immunized with *B. abortus* Rev I live vaccine

Influence of age on the immune response

The results depicted graphically in Fig. 6 show that there is no profound difference based on age in the overall immune response of guinea-pigs. In the group given *B. melitensis* Rev I live vaccine, 4-week-old guinea-pigs gave a poorer MMI value than older ones but they were nevertheless solidly immune.

As in the previous experiment, there was a general association between MMI and immunity, while those animals which were given *B. melitensis* Rev I antigen and showed a high agglutinin titre but weak MMI, were only feebly resistant to infection.

Immunization with Duphavac 45/20 vaccine

Guinea-pigs immunized with Duphavac 45/20 vaccine exhibited fair MMI and appreciable agglutinin levels, but were not solidly immune (Fig. 7). In addition, guinea-pigs given both Duphavac 45/20 and *B. melitensis* Rev I live vaccine were even less immune despite a marked level of MMI and high agglutinin titres.

MMI and immunity in individual guinea-pigs

The spleen mass of immunized guinea-pigs 6 weeks after infection and their percentage MMI were plotted against one another (Fig. 8). There is a very distinct grouping of the immunized animals compared with the controls. With the exception of 1 individual, all the immunized guinea-pigs had an MMI index of over 25% while only 1 of the non-immunized control animals was slightly above this level.

Discussion

By using a non-toxic, crude, soluble antigen extract at a concentration of 25–50 μg protein/ml in the medium, it was possible to establish a satisfactory procedure for assaying MMI in *Brucella* immunized guinea-pigs. Pre-incubation of PEC with antigen did not appear to have any advantage.

Since it is known that the antibody response after immunization against brucellosis wanes with time while immunity persists, guinea-pigs were tested 6 months after immunization in the hope that at this stage they would have a low agglutinin titre but would show a marked degree of MMI and be solidly immune. This proved to be partially true. They were solidly immune but still had a low agglutinin response, while the percentage MMI was also low. A distinct correlation between MMI and immunity could therefore not be established. None the less the correlation, when compared with that of animals with a minimal MMI level, a comparatively high antibody titre and poor immunity, became more apparent.

This idea was further extended by the use of young guinea-pigs, but it was found that even 4-week-old guinea-pigs develop an agglutination titre comparable to that of older animals, while their MMI is poorer. Chen & Elberg (1970) have also shown that guinea-pigs respond very well serologically to *B. melitensis* Rev I vaccine, and it is unlikely that an experimental situation can be established in this species with *B. melitensis* Rev I vaccine where the animals are immune in the absence of specific serum antibodies.

Although Duphavac 45/20 is supposedly non-agglutinogenic (Roerink, 1966), it was found that, in guinea-pigs at least, this is not the case, and similar results have been recorded in cattle by Worthington, Horwell, Mulders, McFarlane & Schutte (1974). Even the use of this vaccine, therefore, did not enable us to achieve our objective of establishing immune animals lacking specific serum antibodies.

In a final attempt to demonstrate a direct correlation between MMI and immunity, the spleen mass of individual immunized animals was plotted against their percentage MMI (Fig. 8). Although a linear correlation could not be established, it is quite evident that, with the exception of 1 animal, all the immunized guinea-pigs had a MMI of 25% or higher. Conversely, except for 1 individual, all the non-immunized animals were below this level. It can thus be concluded that the percentage MMI is closely associated with immunity while there is no association between the agglutinin titre and immunity (Fig. 5).

There are several possible reasons for the absence of a linear correlation between the percentage MMI and immunity.

Firstly, a crude antigen was employed and many antigens, probably operate in the MMI test, react with lymphocytes which are irrelevant to protection, and thus give a misleading result. The use of a purer antigen in the test system is therefore a prerequisite for definitive assays. Similarly, it has been shown that only certain lymphocytes are operative in the MMI system (Pick, 1973) and the use of a purer cell population should also increase the specificity of the test. It is also important to note that species differences occur and that correlates for cellular immunity which are valid in one species are not necessarily applicable in another (Curtis & Hersh, 1973).

Furthermore, since the procedure for assaying immunity that we followed was rather crude and a marked variation among individual animals within a group existed, a more accurate procedure is indicated (Thornton & Muskett, 1972). If graded doses of vaccine are used for immunizing groups of guinea-pigs and these are exposed to different levels of infection, better quantitative results could possibly be obtained. Such elaborate and refined tests, however, require larger numbers of animals and make processing difficult when their MMI properties have to be assayed simultaneously. However, this problem can possibly be largely overcome by the use of an inbred strain of guinea-pigs in which the immune responses would conceivably be more consistent.

Further investigations employing more refined reagents and a more sophisticated assay procedure are definitely indicated.

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