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Inability of Passively Acquired Antibody to Protect Lambs Against Experimental Pasteurellosis

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An experimental model of pneumonic pasteurellosis in sheep was used to investigate the role of serum antibody in resistance to this disease. Lambs which had been vaccinated with a sodium salicylate extract of *Pasteurella haemolytica* type A1 were protected against challenge with PI3 virus followed by *P. haemolytica* type A1 7 days later. The majority of untreated lambs and lambs which had received either 200 ml of antiserum to *P. haemolytica* or 200 ml of control serum intraperitoneally 18 h before infection with *P. haemolytica* type A1 succumbed to the challenge. Lymphocytes from vaccinated lambs showed a specific proliferative response when exposed to *P. haemolytica* type A1 sodium salicylate extract, and this response increased after exposure of these animals to *P. haemolytica* type A1 in aerosol. The results indicate that the humoral immune response alone is incapable of affording protection against experimental pasteurellosis and that cell-mediated immunity may play an important part in resistance to this disease.

The development of an experimental model of pneumonic pasteurellosis in specific-pathogen-free (SPF) lambs (6) has provided a more dependable means of assessing protective immunity conferred by *Pasteurella* vaccines. Previously, assessment of the efficacy of vaccines to *Pasteurella haemolytica* was based largely upon serological criteria, in particular upon the indirect hemagglutination (IHA) test for the detection of antibodies. However, there is no evidence to suggest that measurement of serum antibody titers to *P. haemolytica* gives any indication of the worth of vaccines in preventing disease in sheep. Correlation has been demonstrated between serum IHA titers in lambs vaccinated against *P. haemolytica* biotype A, serotype 1 (type A1) and the protective capacity of these sera when given passively to mice (N. J. L. Gilmour, D. A. Thompson, and D. Mould, manuscript in preparation). However, the resistance of individual vaccinated lambs to challenge with *P. haemolytica* type A1 does not correlate with either of these parameters. Consequently, the role of humoral immunity in protection against *P. haemolytica* infection is unclear.

This paper describes an investigation of the efficacy of passively acquired antibody to *P. haemolytica* type A1 on pneumonia induced by the challenge of SPF lambs with a combined infection of parainfluenza type 3 (PI3) virus and *P. haemolytica* type A1 (6). Data of specfiic proliferative responses of peripheral blood lymphocytes are also presented.

MATERIALS AND METHODS

Sheep. Twenty-seven hysterectomy-derived, colostrum-deprived lambs were reared under SPF conditions. These lambs were allocated to two groups of eight lambs, a third group of seven lambs, and a fourth group of four lambs.

Vaccine. Sodium salicylate extract (SSE) from *P. haemolytica* type A1, prepared as described elsewhere (Gilmour et al., manuscript in preparation), was adsorbed onto aluminium hydroxide gel (Alhydrogel, Superfos, Copenhagen, Denmark) and emulsified in Bayol F (Esso Ltd., Linden, N.J.) containing 10% Arlacel A (Sigma Chemical Co., St. Louis, Mo.) (P. W. Wells, N. J. L. Gilmour, C. Burrells, and D. A. Thompson, Res. Vet. Sci., in press). The same batch of vaccine was used in preparation of antiserum and in the vaccination of lambs for subsequent challenge.

Serology. Serum samples collected from the lambs were tested for antibody to *P. haemolytica* by means of the IHA test (7). The antiserum pools were tested for the presence of hemagglutination-inhibiting antibodies to PI3 virus (8).

Antiserum. A pool of antiserum to *P. haemolytica* type A1 was prepared from blood collected from four colostrum-deprived lambs. These lambs were reared for the first 2 weeks of life under gnotobiotic conditions and then transferred to a room which had previously been thoroughly cleaned and disinfected. The risk of

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cross-infection of these lambs from contact with conventionally reared animals was excluded by their management. *P. haemolytica* type A1 vaccine prepared as described above was injected intramuscularly on three occasions with the second and third injections given 1 and 2 months, respectively, after the first. Blood samples were taken at weekly intervals, and the serum titer of IHA antibody to *P. haemolytica* type A1 was determined. On the basis of these results, blood for preparation of the serum pool was collected over the course of the 2 weeks before and the 4 weeks after the final injection of vaccine. After separation from the clot by centrifugation, the serum was clarified by filtration and sterilized by passing through a 0.22-μm membrane filter. The antiserum pool had an IHA titer to *P. haemolytica* type A1 of 1,024 and lacked antibodies to PI3 virus. A negative control pool of serum obtained from 4- to 6-week-old SPF lambs was prepared in a similar manner. No antibodies to *P. haemolytica* type A1 or PI3 virus were detected in this serum pool.

Treatments. The treatments are summarized in Table 1. At 2 weeks of age the eight lambs in group 1 were vaccinated with 2 ml of the *P. haemolytica* vaccine given subcutaneously over the sternum. Lambs in groups 2 and 3 were not treated at this time. Three weeks later the lambs in all three groups were inoculated intratracheally and intranasally with $10^{7.5}$ 50% tissue culture infective dose ($TCID_{50}$) PI3 virus (10). Six days after infection with PI3 virus, the lambs in group 2 each received 200 ml of antiserum to *P. haemolytica* type A1 and the lambs in group 3 each received 20 ml of control serum. In each case, the serum was administered by intraperitoneal injection in two doses of 100 ml, the second given ca. 6 h after the first. Eighteen hours after the second injection, blood samples were collected from all lambs before groups 1, 2, and 3 were exposed to an aerosol of *P. haemolytica* type A1 (3) which contained 3×10^3 organisms per liter. The lambs in group 4 were neither vaccinated nor infected with either PI3 virus of *P. haemolytica*.

Lymphocyte cultures. Peripheral blood lymphocytes from four lambs in both the vaccinated and unvaccinated groups were obtained and cultured before vaccination and thereafter at intervals during the 4-week period until exposure to the aerosol of *P. haemolytica* type A1. Further cultures were prepared from the same lambs immediately before infection with *P. haemolytica* type A1 and 3 and 5 days after infection. The lambs in group 4 which were not infected with either PI3 virus or *P. haemolytica* were sampled on these latter three occasions. Lymphocytes were prepared and cultured as described previously (1), with the exception that the culture medium contained 5×10^{-5} M 2-mercaptoethanol (4) and cultures were maintained for a period of 5 days in culture medium supplemented with 20% autologous plasma. Lymphocyte proliferative responses to *P. haemolytica* type A1 were determined by the addition of a dilution of SSE to the culture wells and were expressed as stimulation indexes (SI): SI = counts per minute due to incorporation of [^{3}H]thymidine into lymphocyte cultures containing *P. haemolytica* antigen/counts per minute due to incorporation of [^{3}H]thymidine into unstimulated lymphocyte cultures.

Clinical observations. Lambs were examined clinically for 2 weeks after inoculation with PI3 virus. A score for the degree of illness was awarded daily to each lamb by a clinician who was unaware of the identities of the groups, to avoid any subjective influence. Lambs were killed if they showed severe respiratory disease.

Pathology and bacteriology. Lambs that died or were killed when ill were examined at necropsy. Surviving lambs were killed 7 to 10 days after challenge with *P. haemolytica* and examined at necropsy. Drawings of lung lesions were made and measures were taken as described previously (11). Portions of lung tissue were fixed in formol saline and sectioned for histopathological examination. Suspensions were made from the remainder of the lung and the number of viable *P. haemolytica* was counted as previously described, (5) and representative colonies were typed.

RESULTS

Serology. Lambs vaccinated with the *P. haemolytica* vaccine responded by the production of antibodies detectable by the IHA test. Maximum titers (geometric mean, 45.2) were observed on the day that lambs were challenged by exposure to an aerosol of *P. haemolytica*.

Only six lambs in group 2 treated with antiserum of IHA titer 1,024 appeared to have absorbed antibody after the intraperitoneal injection because in two lambs no serum IHA antibody titer was detectable. The geometric mean of the serum IHA antibody titers in these six

TABLE 1. *Summary of treatments given to lambs*[a]

Group	No. of lambs	Treatment at age (days):			
		14	35	41	42
1	8	Vaccinated	Infected with PI3	NT	Infected with *P. haemolytica*
2	8	NT	Infected with PI3	Antiserum i.p.	
3	7	NT	Infected with PI3	Negative serum i.p.	Infected with *P. haemolytica*
4	4	NT	NT	NT	NT

[a] NT, Not treated; i.p., intraperitoneally.

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lambs was 35.9 at the time of infection with *P. haemolytica*. This value was not significantly different from that of the vaccinated group.

Lymphocyte transformation responses. The responses of lymphocytes from vaccinated and unvaccinated lambs to *P. haemolytica* type A1 antigen are shown in Fig. 1. Lymphocytes from the unvaccinated control lambs in group 3 did not respond to stimulation with *P. haemolytica* type A1 until after challenge, when the mean SI increased to 3.4 and 3.3 on days 3 and 4, respectively, after infection. No response to *P. haemolytica* type A1 was observed in lymphocytes cultured from four of the vaccinated lambs before vaccination or 3 days after vaccination. However, by day 7 after vaccination, the mean SI was 3.4 and remained at a similar level during the next 4 weeks of sampling until the time of challenge with the aerosol of *P. haemolytica* type A1. Within 1 day of challenge, the mean SI had begun to rise markedly, to reach a value of 17.5, 5 days after challenge.

Clinical responses. Lambs in groups 1, 2, and 3 which were infected with PI3 virus developed the clinical signs of pyrexia, dyspnoea, anorexia, and dullness described previously as associated with this infection (11). After exposure to the aerosol of *P. haemolytica,* lambs in the vaccinated group showed no sign of clinical illness except a slight pyrexia in two individuals on the day after infection. In contrast, four of the seven lambs in group 3 treated with negative control serum were dull and seven of the eight lambs in group 2 treated with antiserum to *P. haemolytica* showed pyrexia and dullness during the 5 days after infection with *P. haemolytica*. Dyspnoea was observed only in the unvaccinated control and antiserum-treated lambs in groups 2 and 3. The two lambs which did not appear to have absorbed antibody in the group treated with antiserum exhibited more marked clinical signs than did the other lambs in this group and died on day 5 after infection with *P. haemolytica*.

Microbiology. *P. haemolytica* type A1 was isolated from pneumonic areas of the lung in two of the eight vaccinated lambs. In contrast, the bacteria were isolated from pneumonic lung tissue from seven of eight lambs in group 2 treated with antiserum and five of seven lambs in group 3. Mean bacterial counts in pneumonic tissue from these lambs in groups 2 and 3 were $10^{7.4}$ (range, $10^{6.0}$ to $10^{9.0}$) and $10^{7.1}$ (range, $10^{5.9}$ to $10^{8.2}$), respectively, compared with $10^{6.4}$ and $10^{5.8}$ in the two vaccinates. *P. haemolytica* type A1 was isolated from apparently normal lung tissue from four of the eight lambs in group 2 (mean, $10^{5.9}$; range, $10^{3.7}$ to $10^{6.5}$) and two of the seven lambs in group 3 ($10^{3.5}$ and $10^{3.7}$).

Pathology. Macroscopic lesions of pneumonia were observed in the lungs of two of the eight vaccinated lambs in group 1, seven of the eight lambs in group 2 and five of the seven lambs in group 3. The extent of lesions present in the vaccinated lambs, as estimated by planimetric measurement of the drawings showing

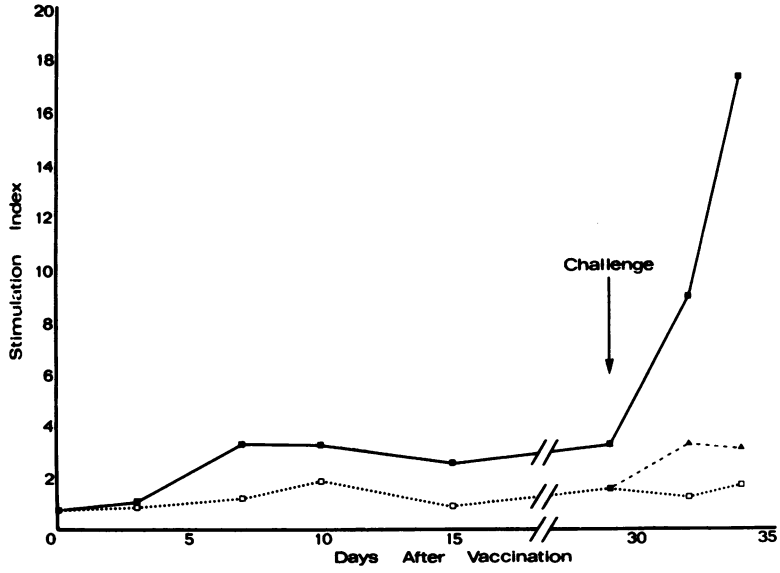

FIG. 1. *Mean SI of groups of sheep in response to vaccination and challenge with P. haemolytica type A1. Symbols: □, Unvaccinated: not challenged (group 4); ▲, unvaccinated: challenged (group 3); ■, vaccinated: challenged (group 1).*

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the pneumonic areas, was significantly less ($P < 0.01$) than in group 2 lambs treated with the antiserum and group 3 lambs receiving negative control serum. There was no significant difference between the extent of pneumonic lesions observed in lambs in the antiserum-treated group and those in lambs in group 3.

Microscopically the pneumonic lesions observed in lambs from all groups were similar to those associated with *P. haemolytica* infection (B. Rushton, J. M. Sharp, N. J. L. Gilmour, and D. A. Thompson, J. Comp. Pathol., in press).

Considering both the results of clinical observation and extent of macroscopic lesions, there was no significant difference between groups 2 and 3. In contrast, the severity of disease assessed in this way in the vaccinated group was significantly less ($P < 0.01$) than in either group 2 or 3.

DISCUSSION

The SSE vaccine of *P. haemolyticca* type A1 has previously been shown to protect against the effects of challenge infection with this serotype (2). In that experiment, lambs were challenged 2 months after vaccination and, in some instances, two doses of vaccine were given. In the present experiment, protection was demonstrated 1 month after a single vaccination. Control lambs exhibited the clinical signs of respiratory disease and at postmortem, pneumonic lesions were more extensive than in vaccinates. Clearly, a vaccine of this type may be of value in the prophylaxis of pneumonic pasteurellosis, and more extensive studies have been carried out with a view to its development (2).

In six of the eight lambs which received antiserum to *P. haemolytica,* serum antibody titers were similar to those recorded in the vaccinated group at the time of challenge. However, no protective effect could be attributed to the antiserum, a finding which supports the contention that serum antibody is not the only factor important in protecting lambs against respiratory disease associated with *P. haemolytica* infection. Previous experiments have shown that serum antibodies are transferred into the secretions of the respiratory tract in sheep (8, 9). Consequently, it is unlikely that any difference between the two groups is due to a difference in the concentration of antibody in the secretions bathing the respiratory mucosa.

It is considered that this experiment implicates cell-mediated immunity as important in the defense mechanisms of sheep which operate to protect against infection with *P. haemolytica* type A1. The data from the lymphocyte transformation studies showed a specific proliferative response in lymphocytes from vaccinates cultured in the presence of *P. haemolytica* type A1 SSE. Such in vitro responses are considered to be a measure of cell-mediated immunity in vivo. Clearly, the proportion of lymphocytes capable of this response appears to increase markedly within a few days of exposure to *P. haemolytica* type A1 as an aerosol. This may be representative of a secondary cellular immune response which is rapid in onset and which could play a valuable part in resisting infection. However, further experiments have indicated that serum from vaccinated lambs has a bacteriostatic effect on *P. haemolytica* in vitro (H. B. Evans, unpublished data) and, consequently, humoral immunity cannot be entirely discounted. Antibody may have a valuable ancillary role in bacterial clearance in vivo. In addition, it may be significant that no antibodies to *P. haemolytica* were detectable in the serum from the two lambs that died in the group treated with antiserum. Indeed, humoral immune responses are significant in protecting against infection with *P. multocida* (13), an encapsulated organism similar to *P. haemolytica.* Experiments are presently being carried out to clarify further the roles of humoral and cellular immunity to *P. haemolytica* in sheep.

It could be concluded from the present results that the monitoring of serological responses may not provide a wholly reliable index of the efficacy of vaccines. However, it would be unwise to disregard serological data completely since there may be some relationship between the humoral immune response and cell-mediated immunity induced by vaccination. In addition, if cell-mediated immune responses are involved in protection, a measure of them may be of value. Further experiments are necessary to clarify this situation, but these results point to the importance of cell-mediated immunity in resistance of sheep to respiratory disease associated with *P. haemolytica.*

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