IMMUNOSUPPRESSION IN NEW-BORN LAMBS

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ABSTRACT

BROEKMAN, J., EKSTEEN, P. A. L. & VERWOERD, D. W., 1977. Immunosuppression in new-born lambs. *Onderstepoort Journal of Veterinary Research*, 44 (3), 151–156 (1977).

A novel technique, based on cytotoxicity-neutralization, was developed for the *in vitro* titration of anti-sheep lymphocyte and anti-sheep macrophage sera. The titres obtained for a number of antisera were compared with those found in an agglutination assay. Anti-lymphocyte sera with a high cytotoxicity-neutralization titre very effectively suppressed the number of circulating lymphocytes in the peripheral blood of treated new-born lambs, thus indicating *in vivo* immunosuppressive activity.

Résumé

L'IMMUNO-SUPPRESSION CHEZ DES AGNEAUX NOUVEAU-NÉS

Une technique nouvelle, basée sur la neutralisation de la cytotoxicité, a été développée pour la titration in vitro *des serums anti-lymphocytes et anti-macrophages de mouton. Les titres obtenus pour plusieurs antiserums ont été comparés a ceux trouvés dans un test d'agglutination. Des serums anti-lymphocytes à un titre élevé de neutralisation de cytotoxicité ont supprimé très efficacement le nombre de lymphocytes circulant dans le sang périphérique d'agneaux nouveau-nés traités, indiquant par là un pouvoir immuno-suppressif* in vivo.

INTRODUCTION

The successful transmission of jaagsiekte (ovine pulmonary adenomatosis) to new-born lambs by intratracheal injection of an established epithelial cell line was recently reported (Coetzee, Els & Verwoerd, 1976). The present investigation of various immunosuppressive techniques was initiated in an attempt to increase the efficacy of transmission and to shorten the incubation period.

Immunosuppression, a standard treatment in transplant patients today, is also commonly used in laboratory animals to increase the efficacy of tumour transplantation (Arnstein, Taylor, Nelson-Rees, Huebner & Lenette, 1974; Sorvari & Arvilomni, 1974). Treatments include thymectomy, the use of corticosteroids, of cytotoxic or immunosuppressive drugs such as cyclophosphamide and azothioprine, and of antilymphocyte serum (ALS) and/or antimacrophage serum (AMS) (Cole & Morris, 1971; Lance & Medawar, 1968; Hirsch, Gary & Murphy, 1969). All these procedures are directed primarily against the cellular immune response in which both lymphocytes and macrophages collaborate in the rejection of transplanted tumours (Zarling & Tevethia, 1973; Piessens, Churchill & David, 1975).

In an extensive study of the effect of thymectomy on immunological responses in the lamb, Morris (1973) found that the rejection of skin transplants was not retarded in 3-month-old lambs thymectomized *in utero* between 60 and 70 days after conception, i.e. when lymphocytes first appear in the thymus. Theilen & Hall (1976), on the other hand, found an enhancement of the growth of transplanted feline sarcoma virus-transformed sheep cells in a thymectomized lamb. Data on the use of immunosuppressants in new-born lambs could not be found in the literature. Some of the most common agents were therefore investigated. Preliminary experiments in this laboratory indicated that corticosteroids have little, if any, immunosuppressive effect in sheep (unpublished results). Results obtained with cyclophosphamide, azothioprine, antilymphocyte and anti-macrophage serum are reported below.

One of the problems encountered in using immunosuppressive sera is the determination of their activity. Various techniques have been used by other workers for the *in vitro* assay of ALS activity. Those based on

cytotoxicity against or on the agglutination of lymphocytes are useful, but there is no direct correlation between the titres obtained and *in vivo* immunosuppressive activity (Martin, 1969). The rosette-inhibition test developed for anti-human lymphocyte serum (Bach, Dormont, Dardenne & Balner, 1969) and the opsonization test used for murine, canine and human ALS (Martin, 1969) showed a better correlation, but neither of these assays yielded satisfactory results with anti-sheep lymphocyte serum in our hands (our unpublished results). A novel method by which neutralization of the cytotoxic action of lymphocytes on a target tumour cell by ALS can be measured was therefore devised and is described in this communication. The technique was also adapted for the titration of AMS, using as indicator cells activated peritoneal macrophages of the mouse which had previously been shown to possess tumour cell specific cytotoxic activity (Krahenbuhl & Remington, 1974; Hibbs, 1974).

Titres obtained by this method were compared with those obtained by means of an *in vitro* agglutination assay, and the *in vivo* activity of ALS estimated in terms of its ability to reduce the number of circulating lymphocytes in lambs.

MATERIALS AND METHODS

Cell cultures

M(52)B murine sarcoma virus-transformed mouse fibroblasts were cultured in Eagle's MEM medium* supplemented with 10% foetal calf serum, using standard techniques. These cells were used as target cells in the cytotoxicity-neutralization assay.

Production of antisera

Anti-sheep lymphocyte serum (ALS) was produced in a horse, using foetal thymus as the source of lymphocytes. Thymuses from mid-term foetuses were minced and the cells mechanically dispersed in MEM tissue culture medium by means of overnight incubation at 4 °C with magnetic stirring. Following the schedule recommended by Fahey (1973), we injected about 10^{10} cells in multiple doses subcutaneously with Freund's complete adjuvant the first time, and followed this with an intravenous injection

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* Tissue culture media were prepared according to the formulations given in the catalogue of Gibco Bio-Cult Ltd, Paisley, Scotland

without adjuvant at 3- and 4-week intervals. Serum was collected 1 week after the last immunization or after further boosters, and stored at —20 °C. Anti-macrophage serum (AMS) was produced in the same way using alveolar macrophages from sheep lungs for immunization. Cells were obtained from 3–4 lungs of freshly-slaughtered normal adult sheep by pouring 1 litre of serum-free F12 medium* into each, massaging the lungs gently and decanting. After the addition of 5% foetal calf serum, cell suspensions were seeded into 10 Roux flasks and incubated at 37 °C for 1 h. Non-adherent cells were washed off and discarded and adherent cells scraped off, suspended in F12 medium and counted with 0,1% trypan blue.

Preparation of immunoglobulin

Immunoglobulin (IgG) was prepared from ALS or AMS by ammonium sulphate precipitation, according to the method of Hebert, Pelham & Pittman (1973).

Agglutination assay

The agglutination assay was based on the method described by Abaza & Woodruff (1966). Lymphocytes and macrophages were prepared in the same way as for immunization, except that, to prevent clumping, polivinyl pyrrolidone (PVP) and heparin were added to the cell suspension at a final concentration of 0,18% and 10 units /ml respectively.

ALS and AMS were inactivated at 56 °C for 30 min before being diluted serially in F12 medium containing PVP and heparin. To 0,1 ml of a serum dilution made on a perspex haemagglutination plate, 0,1 ml of a previously prepared suspension of lymphocytes or macrophages at a concentration of 5×10^7/ml was added and the 2 constituents were gently mixed. The plate was then incubated for 1,5 h at 37 °C in a moist atmosphere containing 5% CO_2. The cells were suspended again by a gentle agitation of the plate, and a few drops examined microscopically. The presence or absence of agglutination was judged from a comparison of the size of the clumps with that of clumps in a parallel dilution series with control (prebled) serum.

Determination of cytotoxicity

Cytotoxicity was measured *in vitro* in terms of inhibition by effector cells of DNA synthesis in target cells, according to a modification of the micro-technique described by Williams, Germain & Benacerraf (1975).

Immune lymphocytes were prepared from sheep immunized at least twice at weekly intervals with subcutaneous or intravenous injections of 1×10^8 M(52)B cells. Fresh blood was collected not longer than 3 weeks after the last injection, and the leucocytes were isolated according to the method of Naylor & Little (1975).

Activated peritoneal macrophages were obtained from mice which were supplied by the Protozoology Section of this Institute and which had been chronically infected with *Toxoplasma gondii* (Droller & Remington, 1975). Peritoneal washings were seeded onto plastic tissue culture dishes and incubated for 1 h at 37 °C. Adherent cells were scraped off, counted, and distributed into the wells of a microtest plate at the concentrations desired.

M(52)B cells were used as target cells at a concentration of 2×10^4 cells/0,1 ml medium per well in Falcon microtest II plates. For the titration of lymphocyte cytotoxicity, the target cells were allowed to attach first, the lymphocytes then being added at various concentrations. Macrophage effector cells were seeded first and allowed to attach, and afterwards the target cells were added.

To measure the cytotoxicity of the effector cells, serial dilutions of immune lymphocytes or activated macrophages were added to a constant number of target cells, as described above, and incubated for 24 h at 37 °C. After the medium had been carefully removed, 0,1 ml of F12 medium containing 0,5 μCi of ^{3}H-thymidine was added to each well in the plate which was then further incubated for another 24 h. After the medium had been decanted, cells were fixed in methanol for 15 min, washed 4 times with methanol and dried for 15 min at 37 °C. Cells in each well were carefully dissolved in 0,2 ml Soluene-350[1], transferred to 8 ml of a toluene scintillation cocktail and counted in a liquid scintillation spectrometer[1].

Cytotoxicity-neutralization assay

Fixed ratios of effector cells: target cells were used for the cytotoxicity-neutralization (CN) assay, as discussed under Results. Effector cells were pre-incubated in serial dilutions of the ALS or AMS for 30 min at 37 °C. In the case of AMS, the macrophages were adherent and the serum dilution could simply be removed before addition of the target cell. When ALS was titrated, the pre-incubation was carried out in suspension and the serum had to be removed by centrifugation before the lymphocytes were added to the already adherent target cells. Further incubation, labelling and processing were the same as for the cytotoxicity tests.

Differential leucocyte counts

Total white cell counts were done on venous blood collected in 0,15% EDTA. The erythrocytes were lysed by diluting 0,1 ml blood into 0,4 ml PBS containing 4 drops of Zap-oglobin[2] before a count was made of the white cells in a haemocytometer.

For differential counts, in which only granulocytes and lymphocytes were differentiated, blood smears were stained with Giemsa.

Immunosuppressive treatment of lambs

ALS or AMS, or the immunoglobulins prepared from them, were administered intraperitoneally in 10 ml doses according to the various schedules mentioned under Results. Ampicillin[3] was added to these solutions at a concentration of 20 mg/ml to prevent bacterial infections. Azothioprine[4] (AZO) were given orally at a daily dose of 4 mg/kg for 14 days. Cyclophosphamide (CY)[5] treatment consisted of 6 daily intravenous injections of 10 mg/kg.

RESULTS

Cytotoxicity of immune lymphocytes and activated macrophages

In order to adapt the micro-cytotoxicity test described by Williams *et al.,* (1975) for use as a neutralization assay, the cytotoxic activity of lymphocytes and macrophages towards the selected target

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* Tissue culture media were prepared according to the formulations given in the catalogue of Gibco Bio-Cult Ltd, Paisley, Scotland

[1] Packard Instruments (Pty) Ltd, Johannesburg
[2] Coulter Electronics S.A. (Pty) Ltd, Benrose, Transvaal
[3] "Pentrex" (Bristol), B.M. Group, Johannesburg
[4] "Imuran", Burroughs Wellcome & Co., London
[5] Koch-Light Laboratories, Coinbrook, Berks., England

cells had first to be determined. The results are shown in Fig. 1. Normal sheep lymphocytes have an inhibitory effect on DNA synthesis at a lymphocyte: target cell ratio of more than 64:1. Immune sheep lymphocytes, by contrast, inhibit 50% of the DNA synthesis at a ratio of 32:1 where normal lymphocytes cause less than 10% inhibition. This ratio was therefore used for subsequent titrations. Activated macrophages have a much stronger inhibitory action on the target tumour cells and an optimum ratio of 2:1 was selected for AMS titrations.

Titration of immunosuppressive antisera by means of the cytotoxicity-neutralization assay

The method used to determine cytotoxicity-neutralization (CN) titres is illustrated in Fig. 2. ^{3}H-Thymidine incorporation was plotted against serum dilution and the 50% inhibition point determined. The reciprocal of the serum dilution corresponding to this point was defined as the CN-titre.

The titre obtained for a series of antisera and the IgG derived from some of them are shown in the first column of Table 1.

Titration of immunosuppressive antisera by means of the agglutination assay

Although the determination of an end-point is rather subjective in this method, reproducible titres were obtained with practice. Titres varied between 640 and 2 560 for ALS and 1 280 and 2 560 for AMS, as shown in Table 1. The fact that the immunoglobulin fractions prepared from both types generally had a lower titre, even though it was usually concentrated 5–7 times, indicates a considerable loss of activity during precipitation.

A reasonable agreement was found between the agglutination titres and the numerically lower cytotoxicity-neutralization titres, also shown in Table 1.

TABLE 1 Titres of various ALS and AMS preparations as determined *in vitro* by means of the cytotoxicity-neutralization and agglutination assays

Antiserum		Cytotoxicity-neutralization titres	Agglutination titres
Control (prebled)		<40	<20
ALS	419	NT	2 560
ALIgG	419	320	1 280
ALS	499	NT	2 560
ALIgG	499	320	1 280
ALS	560	640	2 560
ALIgG	560	320	1 280
ALS	645	480	1 280
ALIgG	645	320	640
ALS	681	480	1 280
ALIgG	681	320	1 280
AMIgG	421	NT	1 280
AMS	487	640	2 560
AMIgG	487	640	2 560
AMS	646	NT	1 280

NT—not tested

Reduction of the blood lymphocyte count as an in vivo *test for immunosuppression*

Differential leucocyte counts were performed on new-born lambs used in transplantation studies with jaagsiekte cell cultures, in order to monitor their reactions to various immunosuppressive treatments. The results are shown in Fig. 3 and 4.
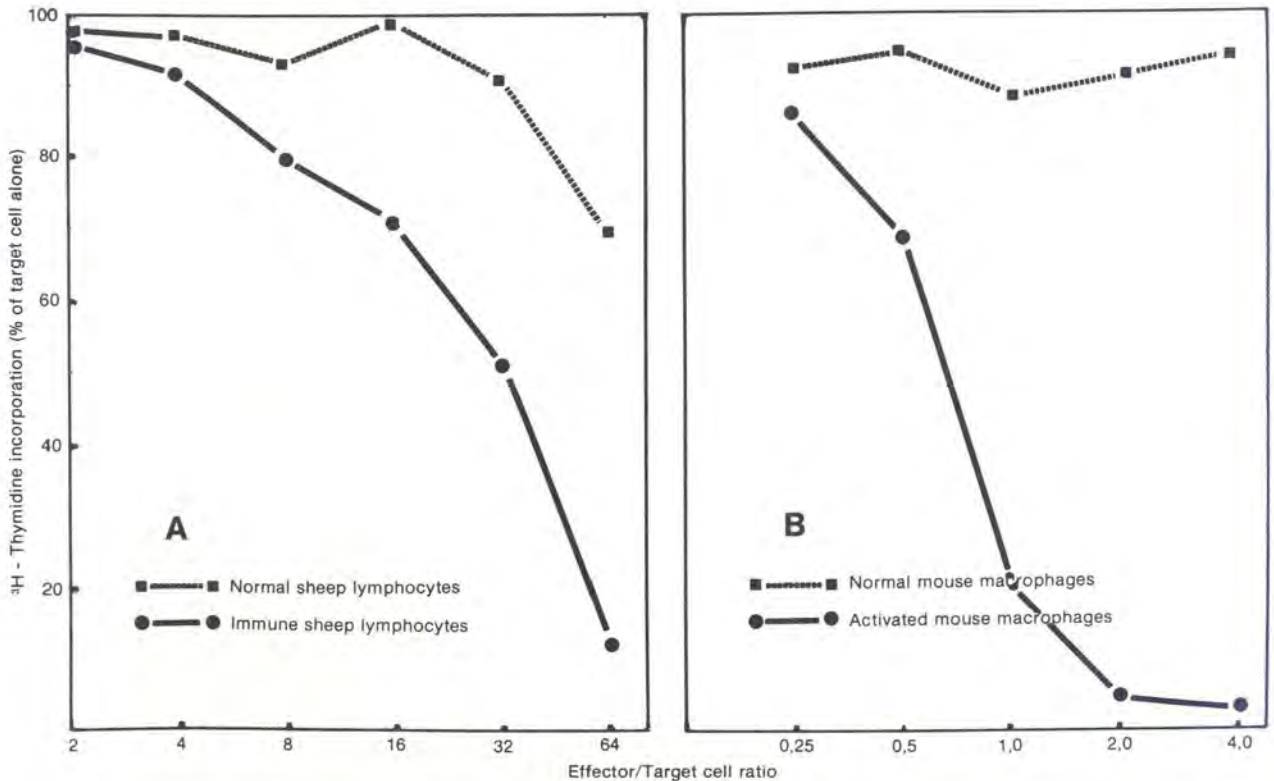

FIG. 1 Cytotoxicity of various ratios of immune sheep lymphocytes (A) and activated mouse macrophages (B) to M(52)B-transformed mouse fibroblasts used as target cells. Cytotoxicity is measured in terms of inhibition of DNA synthesis
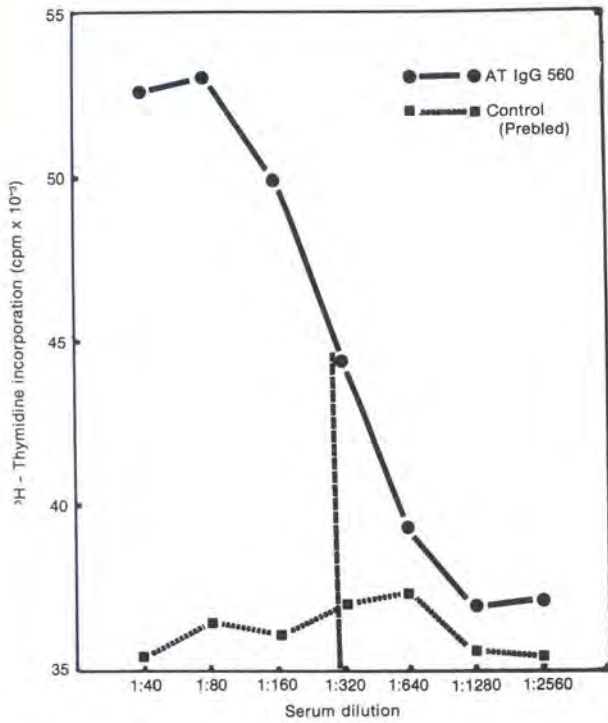
FIG. 2 Determination of the cytotoxicity-neutralization titre of ATIgG(560). Immune lymphocytes were pre-incubated for 30 min at 37 °C with serial dilutions of a control serum and the immunoglobulin before adding to the target cell at a ratio of 32:1 and measuring ³H-thymidine incorporation
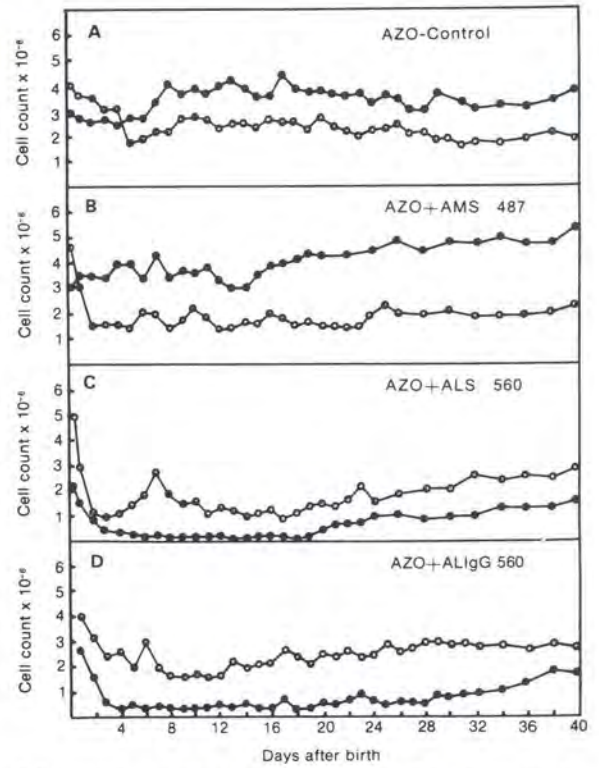

FIG. 3 Leucocyte counts in jugular blood of lambs treated daily for 2 weeks with azothioprine (AZO) alone (4 mg/kg *per os*) (A), azothioprine+antimacrophage serum (AMS) (B), azothioprine+antilymphocyte serum (ALS) (C) and azothioprine+antilymphocyte immunoglobulin (ALIgG) (D). Ten ml of serum or IgG were given intraperitoneally. Granulocytes O——O, lymphocytes ●——●
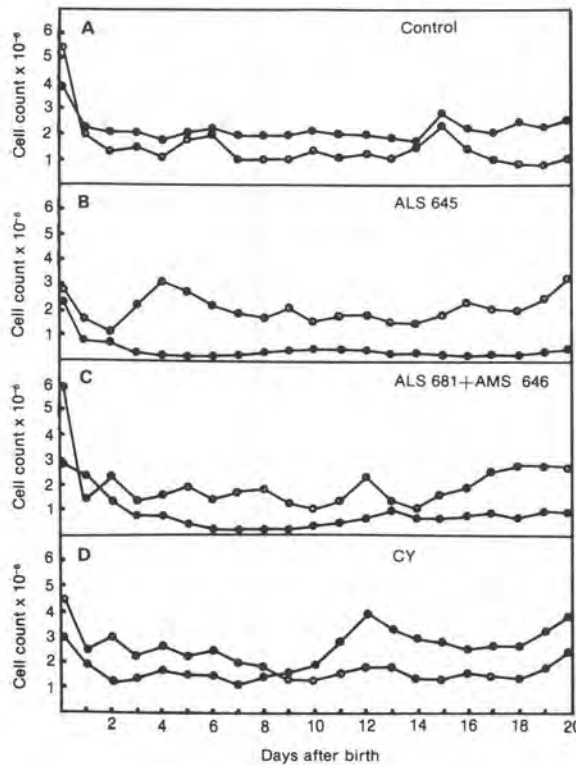

FIG. 4 Leucocyte counts in jugular blood of lambs untreated (A); treated daily for 3 weeks with antilymphocyte serum (ALS) (B); antilymphocyte plus antimacrophage serum (ALS+AMS) (C); and with 6 daily intravenous doses of 10 mg of cyclophosphamide (CY) per kg body mass (D). Ten ml of ALS or of a 50/50 mixture of ALS and AMS were given intraperitoneally. Granulocytes O——O, lymphocytes ●——●

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Fig. 3 represents an experiment in which all the animals were treated for 2 weeks with azothioprine alone or in combination with AMS, ALS or ALIgG. Azothioprine alone had no discernible effect on the leucocyte count. Fig. 3 A can therefore be regarded as a control curve for 3 C-D. AMS did not affect the count, whereas both ALS and ALIgG reduced the lymphocyte counts by about 90% from an average of 3×10^6/ml to between 0,2 and $0,5 \times 10^6$/ml. After treatment was terminated, the lymphocyte count increased gradually, but 4 weeks later it was still well below that of the controls. The reduction obtained with IgG was generally somewhat less than that with the corresponding serum.

In the experiment illustrated in Fig. 4, the effect of ALS alone or in combination with AMS was compared with that of cyclophosphamide. In this experiment the animals received daily intraperitoneal injections of ALS or ALS+AMS for 3 weeks. Treatment with ALS alone yielded results similar to those in the previous experiment. Cyclophosphamide treatment for 6 days reduced the lymphocyte count but to a lesser extent than the ALS treatment, and recovery was complete a few days after cessation of the treatment. In addition, undesirable side-effects, such as complete loss of wool, occurred. The combination of ALS and AMS did not increase the effect on the lymphocyte count of ALS given alone.

DISCUSSION

The role of the cellular component of the immune response in the rejection of tumour grafts is well established, and antilymphocyte serum is widely used to enhance the efficacy of tumour transplantation in experimental animals (Sorvari & Arvilomni, 1974; Arnstein et al., 1974). Since the essential purpose of the present study was to enhance the transmission of jaagsiekte to new-born lambs, the emphasis was therefore mainly on the use of ALS, although various other immunosuppressive reagents including AMS were also investigated. Recently much attention has been focused on the role of macrophages in the cellular response against malignant cells both in vitro and in vivo (Currie & Basham, 1975; Mansell, Ichinose, Reed, Krementz, McNamee & Diluzio, 1975). The use of anti-macrophage serum as a possible means of promoting tumour growth has not yet received the same attention, however.

When ovine material was used, in vitro tests for the activity of ALS developed for human and murine systems, such as the rosette-inhibition and opsonization assays (Bach et al., 1969; Martin, 1969), proved unsuccessful, and this led to the development of a novel method for the in vitro assay of ALS and AMS. This technique was based on the micro-assay for cytotoxicity in which inhibition of DNA synthesis in a target cell is used as a measure of the cytotoxic activity of immune lymphocytes or activated macrophages as effector cells. By pre-incubating the effector cells in dilutions of ALS or AMS, the activity of the latter could be titrated in terms of cytotoxicity-neutralization. The advantage of this method is that the cytotoxicity of the effector cell, which is used as indicator in the titration, is directly related to its role in the rejection reaction, whereas other characteristics, such as its ability to agglutinate the target cell in vitro, are not necessarily related. Reproducible titres, showing fair agreement, were nevertheless obtained with both the CN- and agglutination assays. This suggests that both in vitro assays can be used to test for immunosuppressive activity in antisera, though

the titres would not necessarily correlate with the ability of the sera to suppress the rejection of tumour cell transplants.

An interesting point about the CN-assay for AMS activity is the fact that anti-sheep macrophage serum is active in neutralizing the tumour-specific cytotoxicity of mouse peritoneal macrophages activated by a chronic Toxoplasma infection. This was of crucial importance to the application of the assay for AMS, as activated macrophages have not yet been obtained from sheep.

Since an in vivo method to measure the specific activity of AMS against alveolar macrophages in the sheep has not yet been devised, an in vivo confirmation of the in vitro activity of AMS used in this study was not possible. On the other hand, counting the circulating lymphocytes in the peripheral blood is a simple and convenient way of determining the in vivo activity of ALS. By this means it was shown that various ALS preparations with high CN- and agglutination titres also possess in vivo immuno-suppressive activity. It was found that a daily dose of 10 ml ALS administered intraperitoneally reduced the lymphocyte count of a new-born lamb by about 90% within 3–5 days and that the lymphocyte count recovered only gradually after the termination of treatment. Experimental lambs did not develop any hypersensitivity reactions after daily injections of unfractionated ALS. The IgG preparations were generally found to have lower in vitro titres, confirming the results of Hebert et al. (1973), who showed that precipitation of IgG from horse serum is invariably accompanied by considerable losses. The use of unfractionated serum was thus indicated.

Treatment of lambs with azothioprine had no effect on the lymphocyte count, whereas cyclophosphamide caused a transient reduction. This, however, was accompanied by undesirable side-effects.

In conclusion, therefore, we established that the lymphocyte component of the cellular immune response of new-born lambs can be suppressed effectively by the use of ALS. Suppression of the macrophage component of the reaction can probably be attained by means of AMS, although there is still no experimental proof for this. The effect of treatment with these agents on the transplantation of jaagsiekte will be reported in a subsequent paper.

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