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ANTIBODY - DEPENDENT CELL - MEDIATED CYTOTOXICITY IN SHEEP

By

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MLANGWA, J. E. D.: *Antibody - dependent cell-mediated cytotoxicity in sheep.* Acta vet. scand. 1985, *26*, 105—119. — The ability of sheep leukocytes to mediate antibody — dependent cell-mediated cytotoxicity (ADCC) and that of sheep serum IgG_1 and IgG_2 to induce ADCC were investigated. Partial characterization of effector cells was attempted. These investigations revealed that ADCC occurs in sheep. With chicken erythrocytes (CRBC) as the target cells, polymorphonucleated cells (PMN), and monocytes, were the most effective leukocytes. Ovine peripheral blood lymphocytes (PBL) also mediated ADCC, and within the PBL population, T-cells were capable of mediating ADCC. The T-cells were obtained by nylon wool fractionation and selective agglutination by peanut agglutinin (PNA) and Helix pomatia agglutinin (HPA). Both nylon wool adherent and non-adherent fractions were active in ADCC, although the former were more active, implying heterogeneity in nylon wool adherence among ovine K-cells. Depletion of B (SIg^+) cells did not affect ADCC activity of the remaining cells. Depletion of Fc^+ cells markedly reduced cytotoxic activity of PBL. Both sheep IgG_1 and IgG_2 anti-CRBC immunoglobulins were able to induce ADCC.

lymphocytes; ADCC; T-cells; K-cells; immunology.

Altered self cells, non-self cells and some parasites can be destroyed non-phagocytically in-vitro by non-sensitized leukocytes in the presence of limited amounts of specific antibodies directed against determinants on target cells or organisms (*Perlmann & Holm* 1969, *David* 1982 and *Schultz* 1982).

This phenomenon, designated antibody-dependent cell-mediated cytotoxicity (ADCC) is considered important in that it may reflect an in-vitro vertebrate defence mechanism against non-self and altered self targets and conversely an important mechanism of tissue injury.

ADCC has been investigated extensively in human and rodent systems (*Bloom & David* 1976, *Pearson* 1978 and *Perlmann &*

Cerottini 1979). There are very few reports on ADCC in domestic animals (*Schultz* 1982). In this communication, the occurrence of ADCC in sheep is reported. Evidence is presented that various sheep leukocytes mediate ADCC and that both sheep IgG_1 and IgG_2 can induce ADCC in sheep.

MATERIALS AND METHODS

Target cell preparation

Chicken red blood cells (CRBC) were drawn from wing veins of 10 to 12 week old birds into syringes containing heparin (100 I.U./ml of blood). The blood was diluted 1 in 20 in RPMI-1640 without serum and 100 µl of the diluted CRBC suspension was labelled with $Na_2\ ^{51}CrO_4$ (Radiochemical, Amersham, England) in 100 µl medium for 1 h at 37°C in a 5 % CO_2 atmosphere. After incubation, the cells were washed 3 times and re-suspended at 10^6 cells/ml in complete RPMI medium (10 % FCS, 1 % glutamine, 0.2 % sodium bicarbonate, 100 µg/ml streptomycin and 100 i.u./ml penicillin) just prior to assays (modified from *Perlmann & Perlmann* 1970).

Effector cells

Sheep PBL: These were prepared from heparinized jugular blood by the carbonyl iron technique (*Mlangwa* 1984 a). Briefly, the blood was mixed with a suspension of carbonyl iron (SF Grade) (Fluka, Switzerland) at a concentration of 10—15 mg/ml blood. The mixture was incubated on a rocking platform at 37°C for 1 h with occasional manual mixing. 7 ml of blood were layered on 3 ml of percoll (sp.gr. 1.083) (Pharmacia, Sweden) and centrifuged at 1000 g for 25 min at 18°C. The PBL layer at the interface was collected, washed once, and any contaminating erythrocytes were lysed with 0.83 % Tris-buffered ammonium chloride. The cells were then washed 3 times in PBS. Where fractionated PBL were used as effector cells, fractionation was carried out as described below, except for effector cells fractionated on nylon wool columns, which were fractionated as previously described (*Mlangwa* 1984 a). Nylon wool adherent cells were isolated as described by *Outteridge et al.* (1981 a).

Preparation of ovine PMN, plastic-adherent and non-adherent cells. PMN were prepared as described by *Fujimiya et al.* (1979). Monocyte enriched sub-population

was prepared according to *Kumagai et al.* (1979) with a few modifications. The petri dishes used had been coated at 4°C overnight with 4—5 ml of heat-inactivated foetal lamb serum (Gibco, U.K.).

Lymphocyte fractionation by selective agglutination to lectins. Cell fractionation was done according to *Emery et al.* (1981) with some modifications. Briefly, to 1—2×10^8 PBL in 0.5 ml PBS, 500 µl of lectin (2 mg/ml) in PBS were added, mixed and incubated for 10 min at 20°C. The cell suspension was then layered on 15 ml of 50 % FCS in PBS and allowed to stand until sedimentation was complete. The top and sedimented fractions were harvested separately. Aggregated cells were freed by incubating them with 0.2 % N-acetyl-glucosamine or 2 mmol/l D-galactose for HP lectin and PNA (EY Laboratories, San Mateo, Ca.), respectively. PBL were treated with neuraminidase to expose HP receptors (*Mlangwa* 1984 a).

Depletion of Fc^+ cells. Heparinized bovine red blood cells (BRBC) were washed 3 times in PBS. The buffy layer was removed after each washing. The packed cells were then resuspended as a 2 % (v/v) suspension in saline. One volume of washed BRBC was incubated with one volume of subagglutinating titer of rabbit anti-BRBC IgG fraction (Cappel Labs., Cochranville, Pa.). Incubation was at 37°C for 90 min with shaking every 20 min. The sensitized erythrocytes (EA) were then washed 3 times in PBS and a 1 % suspension of EA in 6 % solution of dextran (T-150) in PBS made (EA_{dex}). One volume of PBL (5×10^6 cells/ml) in RPMI-1640 was mixed with one volume of 1 % EA_{dex} and incubated at 37°C for 90 min in a 5 % CO_2 atmosphere. After incubation, the mixture was layered over Percoll (Pharmacia, Sweden) sp. pr. 1.083 and centrifuged at 1000 g for 25 min at 18°C. Both the interphase and pellet cell fractions were recovered.

The erythrocytes in the pellet and those contaminating the interphase cells were haemolysed with 0.83 % Tris-ammonium chloride solution and washed 3 times in RPMI-medium, checked for viability, counted and adjusted to 10^7 cells/ml for use.

Depletion of SIg^+ (B) cells. 800 µl of packed cell volume (PCV) of washed BRBC were mixed with 800 µl of an IgG fraction of rabbit antisheep IgG antibody (Cappel Labs., Cochranville, Pa.) in a tube. 0.5 % of matured chromium chloride,

pH 5 in saline was then added dropwise while gently whirlmixing the contents. The tube was then slowly rotated by hand for 5 min, after which 3 ml of RPMI-1640 containing 5 % FCS were allowed to run along the side of the tube without rotating it. The cells were then incubated overnight at 4°C and subsequently washed at 200 g for 10 min at 4°C in RPMI-1640-5 % FCS and stored (modified from *Coombs et al.* 1977 and *Ling & Richardson* 1981).

Before use, the sensitized cells (SIg-E) were washed once. 3×10^8 PBL, in 100 ml of RPMI-1640-2 % FCS were mixed with 500 µl of SIg-E (PCV) incubated at 20°C for 15 min, centrifuged at 200 g for 5 min at 18°C and left for 15 min at 20°C. The pellet was resuspended, and fractionation of SIg^- (interface) and SIg^+ (sediment) cells was achieved as described for the depletion of Fc^+ cells. 28 % of PBL formed rosettes.

Sheep anti-chicken red blood cell (CRBC) serum

Anti-chicken red blood cells serum was collected from adult sheep immunized weekly for 3 weeks by injecting 5 ml of packed CRBC in PBS intravenously. The animals were bled 1 week after the last injection. The serum was heat inactivated (56°C for 30 min) and stored at —20°C. The serum agglutinated CRBC in a slide test.

DEAE-Sephacel ion exchange chromatography

DEAE-Sephacel ion exchange chromatography was carried out according to *Aalund* (1968), with some modification. The sheep anti-CRBC serum was dialysed for 12 h against 0.01 mol/l sodium phosphate buffer at pH 8. 3—5 ml of the dialysed serum were then chromatographed on a 1.5×28 cm DEAE-Sephacel (Pharmacia, Uppsala, Sweden) bed equilibrated with 0.01 mol/l phosphate starting buffer (pH 8) and 0.3 mol/l sodium phosphate limiting buffer (pH 8), respectively.

Mixing was achieved by a magnetic stirrer in the vessel containing the starting buffer. 3—4 ml fractions were collected (LKB-Fraction Collector, Sweden) at a flow rate of 20 ml per h.

Sephacryl S-300 gel chromatography

Samples were dialysed for 24 h with 2 changes of 0.01 mol/l sodium phosphate buffer at pH 8. A 2.5×75 cm column was used with a sephacryl S-300 (Pharmacia, Uppsala, Sweden) bed height

of 66 cm, equilibrated with 0.1 mol/l Tris-buffer at pH 7.8 containing 1 mol/l sodium chloride. 3—4 ml samples were applied and eluted with the equilibration buffer at a flow rate of 20 ml per h, collecting 3—4 ml fractions (modified from *Aalund* 1968).

Protein concentration

Protein was concentrated by negative pressure ultrafiltration using Sartorius membrane filtration (Colloidion Bags, SM 13200, G.m.b.H., Gottingen, Germany) or by reverse osmosis, the samples in dialysis tubes were immersed in 40 % poly-ethylen-glycol 7—20000 until the desired volume was reached.

Protein concentration determination was made using a Spectronic-21S (Bausch & Lomb, U.S.A.) spectrophotometer. Absorbance (OD) at 280 nm was measured.

Immunoelectrophoresis

Immunoelectrophoresis was carried out according to *Aalund* (1968), with some modifications. Briefly, 3 ml of 1 % Noble agar (Difco Labs., Detroit, Michigan, U.S.A.) in Gelman buffer (pH 8.6, conductivity 3.1 ms) were poured on levelled microscope slides (26×76 nm). On solidifying, wells and antiserum trenches were punched using an LKB gel punch set. 2.5—3 µl of sample were applied to the wells, after the agar plugs had been sucked off with a 19G levelled needle connected to a suction system.

Electrophoresis was run for 90 min at 6 volts per cm. Then, antiserum troughs were prepared by removing the cut gel using a needle, and rabbit anti-sheep IgG serum was poured into the troughs. Immunodiffusion and precipitation was developed at 20°C in a humid chamber for 18 to 24 h.

Cytotoxicity assay

To 10×75 mm polystyrene test tubes, various numbers of washed effector cells in 100 µl complete medium were added, followed by 100 µl of target cells (10^5 CRBC/tube) and finally 100 µl of appropriate inducer molecule. Rabbit IgG fraction of anti-CRBC or anti-BRBC serum (Cappel Labs., Coachranville, Pa.) and sheep anti-CRBC serum or its IgG fractions were used.

The tube contents were mixed and incubated at 37°C in a humidified atmosphere containing 5 % CO_2 for 20 h, except where indicated (cf. Fig. 3).

After incubation and addition of 700 µl of cold PBS to each tube, the tubes were centrifuged. 500 µl of the supernatant fluid were removed for counting.

Data presentation

Per cent cytotoxicity (% ^{51}Cr release) was calculated according to the formula:

% cytotoxicity = 100 × (experimental release counts — control release counts)/(maximum release counts — control release counts);

where experimental release counts = counts in supernatants containing effector cells + target cells + specific or non-specific antibody molecules;

control release counts = counts in supernatants containing effector cells + target cells only;

maximum release counts = counts in supernatants from target cells incubated with a 5 % Triton X detergent. An additional control was included: target cells in media with sensitizing antibodies but no effector cells. This gave the same counts as supernatants of target cells in media alone.

RESULTS

Demonstration of ADCC to CRBC in sheep

Sheep PBL were found to destroy antibody sensitized CRBC. The PBL exhibited cytotoxicity within the range 19—30 %, the reaction being detectable at an effector-to-target cell ratio of 25:1 (Fig. 1). In the absence of rabbit anti-CRBC IgG antibody, the cytotoxic activity of the effector cells was similar to that of target cells alone (spontaneous release).

Addition of rabbit anti-bovine red blood cell IgG fraction to the assay instead of rabbit anti-CRBC IgG fraction gave results, which were similar to the effect of anti-CRBC IgG fraction on target cells alone in which cytotoxic activity was negligible (data not shown).

Very low levels of specific antiserum were sufficient to induce detectable reaction (Fig. 2). Maximal cytotoxicity was found at antiserum dilutions between 1:2500 and 1:12500. At higher serum concentrations, prozone effect was evident. At lower serum concentrations, cytotoxicity was reduced.

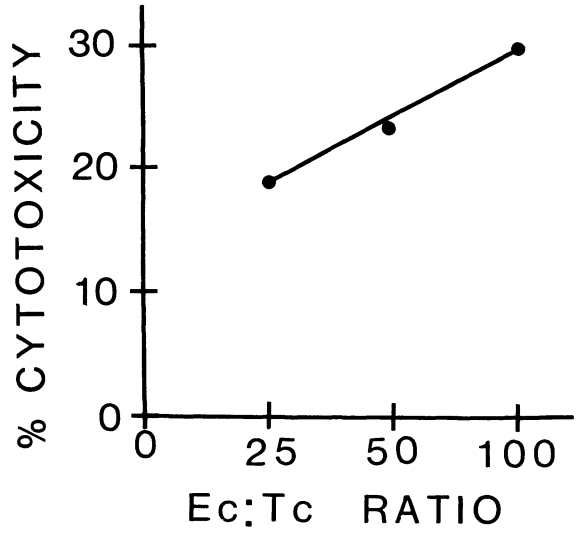

Figure 1. Effect of effector-(E_c) to-target (T_c) ratio on ADCC. Rabbit anti-CRBC IgG serum fraction was used at a dilution of 1:500.

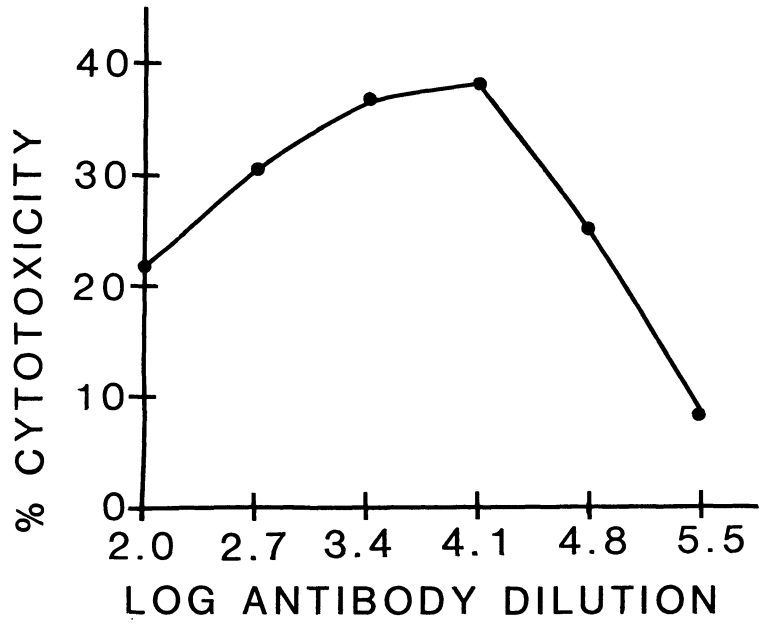

Figure 2. The effect of rabbit anti-CRBC IgG concentration (log ab dilution) on ADCC. An effector-to-target cell ratio of 50:1 was used.

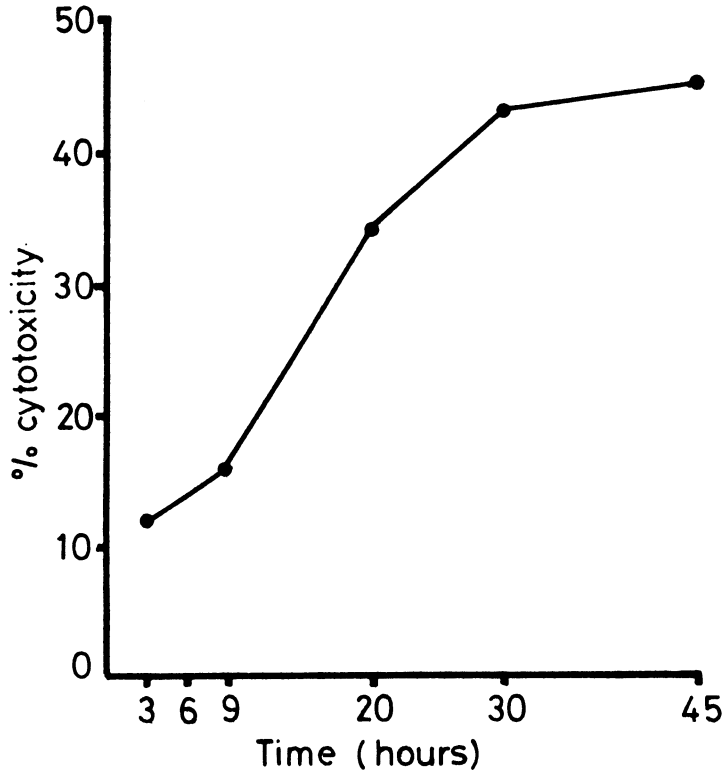

Figure 3. ADCC kinetics. Effector cells (PBL) were incubated with target cells (CRBC) at an effector-to-target cell ratio of 100:1 in the presence of rabbit anti-CRBC serum diluted 1:2500.

In-vitro kinetics of the ADCC reaction effected by PBL showed that although a 20 h period of incubation was convenient to detect specific lysis, a period of 30 to 45 h of incubation was required for optimal lysis (Fig. 3).

Characterization of cells mediating ADCC

The effector cells used in the preceding section were composed of 98.6 % lymphocytes with less than 1 % latex particle ingesting cells (*Mlangwa* 1984 a). Since phagocytic cells were removed during PBL preparation, the ability of polymorphonucleated cells (PMN) and plastic adherent cells (AD) (mainly monocytes) to mediate ADCC were also tested. PMN, AD, and non-adherent cells were all very effective in mediating ADCC against CRBC (Table 1).

Table 1. Cytotoxic (ADCC) activity of polymorphonucleated cells (PMN), plastic adherent (AD) and non-adherent (NAD) cells against CRBC.[a]

Effector cells				
population	purity %	criteria	EC:TCb	% cytotoxicity
PMN	96	Acridine orange staining morphology	18:1	96
AD	63	cells ingesting ≥ 3 latex particles	16:1	92
NAD	83	cells ingesting < 3 latex particles	100:1	84

[a] Rabbit anti-CRBC antibody at a dilution of 1:2500 was used.
[b] EC : TC = effector-to-target cell ratio.

All 4 fractions of PBL separated by selective agglutination to HP and PNA lectins were cytotoxic (Table 2).

Table 2. ADCC activity of various PBL fractions separated by selective agglutination to lectins (PNA and HP). The effector-to-target cell ratio was 100:1, and rabbit anti-CRBC was used at a dilution of 1:2500.

Effector cell population	PBL	PNA+	PNA−	HP+	HP−
% Cytotoxicity	50	56	37	47	44

\+ agglutinated fractions.
— non agglutinated fractions.

Table 3. ADCC activity of lymphocytes separated on nylon wool columns.

Effector cell Fraction	% cytotoxicity		
	Experiment 1		Experiment 2
	EC : TC ratio		
	100 : 1	50 : 1	100 : 1
PBL	26	25	30
Adherent[b]	48	—[a]	46
Non-adherent[b]	14	11	17

EC : TC ratio = effector-to-target cell ratio.
[a] not tested.
[b] significantly different from PBL ($P < 0.05$).

Table 3 shows the cytotoxic activity of cells separated on nylon wool columns. In both experiments and at all target-to-effector cell ratios tested, the nylon wool adherent sub-populations were more efficient in mediating ADCC than PBL ($P < 0.05$). The non-adherent fractions retained a substantial cytotoxic activity, which was significantly reduced ($P < 0.05$) compared to the PBL activity.

Depletion of F_c^+ cells reduced the level of cytotoxicity by 71 %, whereas depletion of SIg^+ cells had little effect ($P > 0.05$), reducing ADCC activity by 13 and 18 % at effector-to-target cell ratios of 100:1 and 50:1, respectively (Table 4).

Table 4. ADCC activity of effector cells fractionated by rosette depletion.

Effector cell	% cytotoxicity		
	rosette depletion technique		
	Fc	SIg	
	100 : 1[a]	100 : 1	50 : 1
PBL	56	28	31
Interface	16	23[b]	27[b]
Pellet	37	NT[c]	NT

[a] Effector-to-target cell ratio.
[b] Not significantly different from PBL ($P > 0.05$), student t-test.
[c] Not tested.

Induction of ADCC by sheep immunoglobulins

Sheep anti-CRBC antiserum induced ADCC in a homologous system, but activity declined rapidly with the dilution of the antiserum.

The sheep anti-CRBC antiserum developed arcs corresponding

Table 5. Induction of ADCC by sheep and rabbit anti-CRBC IgG fractions.

IgG source	Anti-CRBC IgG fraction	% cytotoxicity
Rabbit	IgG	37
Sheep	IgG_1	26
	IgG_2	29
	* Unfractionated serum	14

An effector-to-target cell ratio of 100:1 was used.

to IgG_1, IgG_2 and IgM in immunoelectrophoretic slides, whereas serum fractionated on DEAE-Sephacel and Sephacryl 300 S columns developed single arcs identified as IgG_1 and IgG_2 according to *Aalund* (1968). Both IgG sub-classes induced PBL-effected ADCC against CRBC (Table 5).

DISCUSSION

ADCC occurs in man (*Branco et al.* 1976), mice (*Ramshaw & Parish* 1976), horse (*Fujimiya et al.* 1979), bovine (*Grewal & Rouse* 1979), dog (*Ho & Babiuk* 1979) and pig (*Binns* 1982). The present results show that non-immune ovine leukocytes in the presence of small amounts of anti-CRBC antibodies mediated ADCC.

In the above-named species, depending on the type of target cells, null lymphocytes, T-lymphocytes, monocytes and PMN leukocytes are able to effect ADCC induced by heterologous or homologous anti-target cell serum or antibodies of the IgG class. Sheep PMN and plastic-adherent mononuclear cells were more efficient in affecting ADCC than PBL. Although the plastic-adherent cells contained both monocytes and lymphocytes, most of the activity can be attributed to the monocytes because, at the effector-to-target cell ratio used (16:1) PBL would have had negligible activity. And in all experiments in which PBL or purified lymphocyte sub-populations were used at higher effector-to-target cell ratios (100:1 and 50:1), maximum activity was never above 56 % compared to a percentage release of 92 % by the plastic-adherent fraction. The plastic dish non-adherent (NAD) population had quite a high activity. This could also be explained by the contamination of the NAD population by monocytes due to intrinsic agglutination (*Outteridge et al.* 1981 b, *Mlangwa* 1984 a, 1984 b).

As in other species (*Perlmann & Cerottini* 1979 and *Grewal & Rouse* 1979), the lymphocytes mediating ovine ADCC express Fc receptors, since depletion of Fc lymphocytes reduced the ADCC activity of PBL markedly. B-cells do not seem to mediate ADCC in sheep, because depletion of cells bearing SIg did not lower K-cell activity significantly. The marginal lowering of cytotoxic activity in SIg^+ cell depleted PBL could have arisen from depletion of K-cells during fractionation as a result of non-specific aggregation of K-cells to SIg^+ rosettes.

In the bovine (*Grewal & Rouse* 1979) and man (*Thorsteinsson et al.* 1977), passage of PBL through nylon wool columns enhances K-cell activity of non-adherent cells against target cells susceptible to lymphocyte effected ADCC. Contrary to these findings, the recoverable nylon wool adherent fractions were more active than both the PBL and non-adherent fraction. Since monocytes and PMN were already depleted from the initial PBL fraction (*Mlangwa* 1984 a) and B-cells probably do not effect ADCC (vide supra), it appears that there is a heterogeneity of ovine SIg^- lymphocytes mediating ADCC with respect to their ability to adhere to nylon wool. The recoverable nylon wool adherent sheep lymphocytes contain a substantial number of T-cells and null cells (*Outteridge et al.* 1981 a and b), and probably it is these cells that efficiently mediate ADCC.

The data suggest that in sheep T-cells mediate ADCC. Nylon wool non-adherent cells, HP^+ and PNA positive fractions were active in ADCC assays. These lymphocyte populations are highly enriched in T-cells and depleted of B-cells, and their responses to mitogens (PHA and LPS) were typical of T-cells (*Mlangwa* 1984 b). Since null cells are thymus dependent in the sheep (*Fahey et al.* 1980) and respond to T-cell mitogens (*Outteridge et al.* 1981 a), they may be regarded as a sub-population of T-cells by definition. In man, it has been established that a significant proportion of ADCC-mediating lymphocytes against nucleated as well as erythrocytic target cells can form spontaneous rosettes with SRBC (*Perlmann & Cerottini* 1979), and most of these E^+ K-cells had receptors for HP agglutinin (*Hellstrom et al.* 1976).

Heterologous (rabbit) anti-chicken erythrocyte antibodies of the IgG class and homologous sheep anti-CRBC antibodies were able to induce ADCC. Although IgM is active in inducing ADCC in some systems (*Lamon et al.* 1977 and *Zoller et al.* 1982), it is the IgG fraction which predominates in inducing ADCC. In man, IgG_1, IgG_2, IgG_3 and, to a lesser extent, IgG_4 sub-classes can induce cytotoxicity depending on the type of effector cells (*Bloom & David* 1976). Sheep has two IgG sub-classes, IgG_1 and IgG_2 (*Tizard* 1982). With ovine PBL as effector cells, both sheep IgG_1 and IgG_2 were able to induce ADCC, implying that sheep K-lymphocytes have receptors for both IgG sub-classes and ADCC probably occurs in-vivo as an immune mechanism.

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SAMMENDRAG

Antistof - afhængigt celle-medieret cytotoksisitet (ADCC) hos får.

Fåreleukocyters evne til effektuering af ADCC blev undersøgt, ligesom IgG_1 og IgG_2 undersøgtes for evne til at inducere ADCC. En delvis karakterisering af effektorcellerne forsøgtes. Disse undersøgelser viste, at ADCC forekommer hos får. Med kyllingeerythrocyter som målceller var ovine polymorfnukleære celler, monocyter, de mest effektive celler. Lymfocyter virkede også ved ADCC, og inden for denne population kunne T-celler effektuere ADCC. T-cellerne var isolerede ved nylonvat fraktionering og selektiv agglutinering med PNA og HPA. Både på nylonvat adhærerende og ikke-adhærerende fraktioner var aktive i ADCC, skønt de førstnævnte viste størst aktivitet. Dette indicerede heterogenitet for nylonvat adhærans i ovine K-celler. Fjernelse af celler med immunglobuliner på cellemembranen påvirkede ikke ADCC aktiviteten af de resterende celler. Såvel IgG_1 som IgG_2 anti-kyllinge-erytrocyt immunglobuliner kunne forårsage ADCC.

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