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QUANTITATION OF IMMUNOGLOBULINS IN OVINE SERA AND SECRETIONS BY LASER NEPHELOMETRY. COMPARISON WITH THE RADIAL IMMUNODIFFUSION (RID) TECHNIQUE

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ABSTRACT

Goudswaard, J., Verdouw-Chamalaun, C.V.M. and Noordzij, A., 1980. Quantitation of immunoglobulins in ovine sera and secretions by laser nephelometry. Comparison with the radial immunodiffusion (RID) technique. Vet. Immunol. Immunopathol., 1: 163-177.

In twenty-five ovine body fluids (serum, lung fluid and cerebrospinal fluid), the concentrations of IgG1, IgG2, IgM and IgA were determined by laser nephelometry and radial immunodiffusion (RID). When nephelometric assays are carried out, antisera free from any turbidity are essential. Methods ensuring that goat and rabbit antisera will satisfy this requirement are described in the present paper. When sheep immunoglobulins were measured by laser nephelometry, adequate and reproducible results were obtained, comparable with those obtained by RID.

Advantages of the nephelometric method include the speed of assay and its sensitivity, allowing precise determination of the very low concentrations of immunoglobulins in CSF.

INTRODUCTION

Although a number of papers on the quantitation of proteins by turbidimetric techniques have appeared in the literature (Ritchie, 1967; Killingsworth and Savoy, 1971), the radial immunodiffusion method (RID) developed by Mancini et al. in 1965 continues to be the method most widely used in estimating immunoglobulin concentrations in body fluids. This situation has altered recently. Although nephelometric techniques in which 90° light scatter is measured, have been used to quantitate proteins in serum and cerebrospinal fluid, the fact that greater total light scatter is detectable by observing

this scatter at a forward angle (Marrack and Richards, 1971) was critical in the development of the nephelometers used today to determine various serum proteins in human subjects. The advantages of observing the scatter at a forward angle include increased sensitivity and an increased difference between the sample blank and immunological reaction in percentages of relative light scatter ratios

The present study was designed to develop antisera for the nephelometric determination of immunoglobulins in sheep, which are satisfactory and yield reproducible results. This paper is concerned with estimation by laser nephelometry and RID of classes and subclasses of ovine immunoglobulins in sera, lung fluid and cerebrospinal fluid (CSF). A limited number of samples from diseased or infected animals were included to enable optimal comparison of the two methods rather than to reach any definite conclusions regarding concentrations of immunoglobulins in particular disease states.

MATERIAL AND METHODS

Sheep sera

All samples were collected from Texel sheep. Four sera (1 - 4) were obtained from normal adult sheep, six (5 - 10) from normal lambs (5 - 9: one day old; 10: five days old), one from an animal with pulmonary adenomatosis (11), the first case to be reported in the Netherlands (Herweijer, 1978), one from a sheep which died showing symptoms of endocarditis and endometritis (12) and six (13 - 18) which were collected annually for six consecutive years from an animal infected by the intrapulmonary route with maedi/visna virus (De Boer, 1970).

Lung fluid

Lung fluid (19) was obtained from the same clinical case of pulmonary adenomatosis by elevating the affected animal posteriorly and collecting the fluid discharged from the nostrils (Smith et al, 1975).

Cerebrospinal fluid

Cerebrospinal fluid was obtained by suboccipital puncture. Localization between the occiput and atlas is simple and the subarachnoid space is readily accessible and wide so that a sufficient quantity (at least 5 ml.) of CSF could be collected. Only completely clear samples free from blood were studied. One sample was collected from the animal with pulmonary adenomatosis referred to previously (20), one from a normal healthy sheep (21) and four were obtained from the four normal sheep 1 - 4 (22 - 25 respectively).

Preparation of Ig class- and IgG-subclass-specific antisera

The antisera were raised in rabbits (IgG1, IgA and IgM) and goats (IgG2) and made monospecific as previously described (Verdouw-Chamalaun et al., 1977). However, for use in nephelometric assays non-turbid antisera are required to obtain low antibody blanks. Therefore, the goat antiserum was delipidated and fractionated by precipitation with 40% saturated ammonium sulfate. The serum then was passed through a DEAE cellulose column (2.5 x 20 cm.) equilibrated with 0.01 M phosphate (PO4) with 0.05 M NaCl pH 7.6. (Goudswaard and Virella, 1977). The excluded protein was dialyzed against .15 M NaCl and concentrated by vacuum dialysis. The protein concentration of this antibody containing fraction, as determined according to Lowry et al. (1951) was 33 mg/ml. Several dilutions of this preparation were tested for use in the nephelometric assay of IgG2 in the samples; a 1:20 dilution with saline and a polymeric buffer[x] being found to be best.

Rabbit antisera could be used after simple affinity chromatography with protein A (Goudswaard et al., 1978). The IgG-containing fractions were eluted with 0.1 M glycine - HCl buffer, pH 2.5, and immediately neutralized with 0.5 M TRIS. The samples then were dialyzed against .15 M NaCl and concentrated by vacuum dialysis. Protein concentrations of the various antibody containing fractions were for anti-IgG1: 9 mg/ml, for anti-IgA: 2 mg/ml and for anti-IgM: 11 mg/ml, and the dilutions of these fractions for the nephelometric assay were 1:50, 1:20 and 1:40 respectively. All Ig class and IgG subclass-specific

[x]Hyland Division Travenol Laboratories Inc., Costa Mesa, California, USA 92628, P.O. Box 22114.

antisera were analyzed by RID as well as by nephelometry with 4 fractions, containing 18.4 mg/ml IgG1 + 1.6 mg/ml IgG2, 11 mg/ml IgG2, 0.775 mg/ml IgA and 1.35 mg/ml IgM + 0.15 mg/ml IgG1 respectively.

Standard samples

A "reference serum" (Verdouw Chamalaun et al., 1977) was used, which contained 19.20 mg/ml of IgG1, 11.20 mg/ml of IgG2, 4.20 mg/ml of IgM and 0.36 mg/ml of IgA. Although the concentrations of various immunoglobulins, particularly IgA, were rather high in this reference serum, it was decided to adopt a different reference in measurement of IgA. For this reason, the lung fluid referred to previously was chosen as a reference. The concentration of IgA, determined by RID and nephelometry with heavy chain specific rabbit anti sheep IgA was 5.57 mg/ml. In this case reference serum was used as a standard.

Laser nephelometry

A nephelometer[x] was used, which incorporates a laser as the light source and an optical system detecting forward light scatter. The method mainly adopted was that described by Deaton et al., (1976), the necessary adjustments being made for the purpose of the present study. The working antisera were filtered through a 0.4 µm. polycarbonate membrane (Nucleopore[xx]) to remove particulate matter and the saline blank was filtered by the same method. For each assay system, 1 ml of the working "antisera" was added to a series of 10 x 75 mm. disposable cuvettes. One ml of the saline was added to a separate series of cuvettes for each reference and unknown blank. A saline blank and antiserum blank were prepared by pipetting 1 ml of saline and antiserum respectively into the cuvettes. The reference sample was used in six different dilutions in saline; for IgG1 as well as for IgG2 most samples could be tested in dilutions 1:100, with the exception of the samples from newborn lambs.

All IgA samples were diluted 1:2 before use and most IgM samples had to be diluted 1:20, whereas all CSF samples were assyed undiluted. Dilutions of standards and unknowns were pipetted into the

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sample blank and test cuvettes (25 µl. for the sera and lung fluid, 100 µl for the CSF). Incubation and measurement of the percentage of relative light scatter were carried out as described by Deaton et al., (1976).

Radial immunodiffusion

Radial immunodiffusion was carried out as previously described (Verdouw-Chamalaun et al., 1977).

Immunoelectrophoresis and Ouchterlony analyses

Immunoelectrophoresis and Ouchterlony analyses were carried out using standard procedures in 1.5 per cent agarose.[x]

Correlation between nephelometry and RID and accuracy of both test methods

On the basis of knowm findings (Deaton et al., 1976; Goudswaard and Virella, 1977) showing that the relative error in observation using the two methods is constant throughout the region, regression was applied to the pairs (x, y), where y = LN (LAS) (LAS = concentration of immunoglobulins as determined by laser nephelometry) and x = LN (RID) (RID = concentration of immunoglobulins as determined by single radial immunodiffusion). In carrying out the calculations, a third degree polynomial ($y = a_3x^3 + a_2x^2 + a_1x + a_o$) was used (v.d. Waerden, 1957) only those coefficients being retained, which differed significantly (P = .05) from 0. These calculations showed that only the IgG1 levels as determined by the two methods were not linear. However, the quantitative contribution of this coefficient was so small that a linear approach with $a_o = 0$, i.e. the LN (LAS) = a_1LN (RID) model, was also maintained in the estimations of IgG1. a_o only differed significantly in estimations of IgM; this coefficient contributed so little that the same model could be maintained for the sake of simplicity.

Finally, the accuracy of the two methods was assessed by assaying eight samples on four consecutive days. Each day the nephelometer was recalibrated, fresh dilutions of samples and antisera were made and the RID plates also were moulded again.

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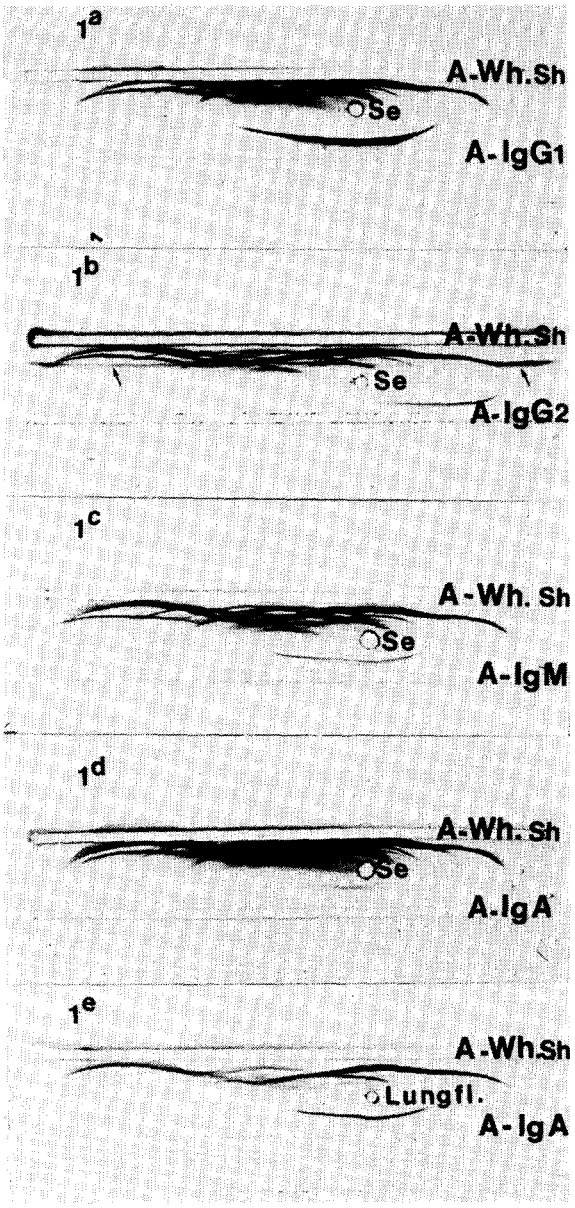

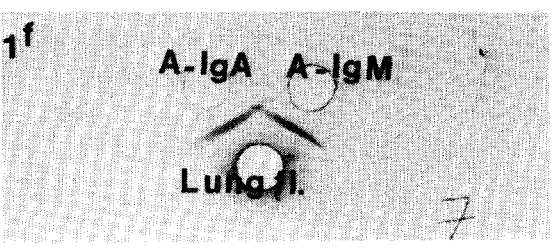

Fig. 1. Immunoelectrophoretic patterns of sheep serum and lung fluid developed with polyvalent and specific antisera. 1a-d: sheep serum in wells developed with polyvalent rabbit anti-whole sheep serum (upper trough) and specific antisera against IgG1, IgG2, IgM and IgA (lower trough). Note at the arrows: The precipitation lines are not absorption lines, but normal immunoprecipitates between goat serum proteins and cross-reacting rabbit anti-sheep serum proteins. 1e: lung fluid of a case of pulmonary adenomatosis developed with polyvalent rabbit anti-whole sheep serum (upper trough) and anti- IgA (lower trough). Anode to the left. 1f: Ouchterlony analysis of lung fluid referred to under 1e with specific antisera against IgA and IgM. No cross-precipitation occurs.

RESULTS

The specificity of the antisera is illustrated in Fig. 1. Fig. 1a shows whole sheep serum developed with rabbit-anti-whole sheep serum and with specific rabbit-anti-IgG1; Figs. 1b, 1c and 1d show goat-anti-IgG2, rabbit-anti-IgM and rabbit-anti-IgA being tested.

In addition, lung fluid used as a standard in IgA determinations was also developed with rabbit-anti-whole sheep serum and rabbit anti-IgA (Fig. 1e). No cross-precipitation occurred between the IgA precipitin line and the IgM precipitin line (Fig. 1f).

Analyses of the antisera with fractions of IgG1, IgG2, IgA and IgM by RID (Verdouw-Chamalaun et al., 1977) and laser nephelometry produced comparable results.

The range of linearity for the nephelometric reagents was determined by plotting the percentage of relative light scatter of a series of reference dilutions as representative concentrations for the immunoglobulin (sub)classes on linear graph paper.

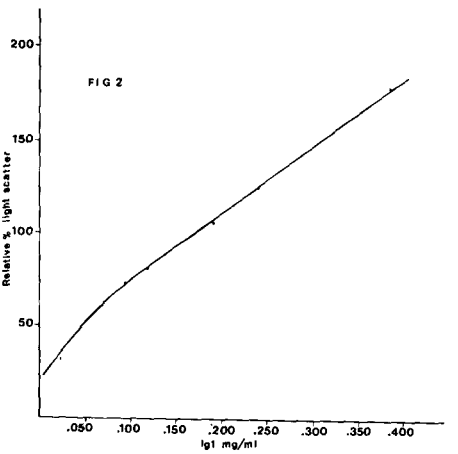

Fig. 2. Calibration curve for the assay of IgG1 by laser nephelometry.

Highly satisfactory calibration curves were obtained. The calibration curve for IgG1 concentrations ranging from 0.384 to 0.024 mg/ml. is shown in Fig. 2 as an example.
All assays could be carried out at instrument sensitivity 2.
Antibody blanks read for the dilutions of "antisera" were 10.7 for

anti-IgG1, 5.5 for anti-IgG2, 20.7 for anti-IgM and 11.6 for anti-IgA.

TABLE 1

Concentrations of IgG1, IgG2, IgM and IgA in mg/ml., as determined by laser nephelometry (LAS) and single radial immunodiffusion (RID).

sample	IgG1		IgG2		IgM		IgA	
	LAS	RID	LAS	RID	LAS	RID	LAS	RID
1	18.82	15.08	12.00	13.00	2.54	3.00	0.184	0.076
2	20.97	16.93	13.70	13.80	2.94	2.80	0.040	trace
3	19.97	22.83	12.50	13.00	3.50	3.80	0.069	trace
4	23.72	21.05	7.12	6.40	2.84	3.20	0.061	0.058
5	34.40	37.95	0.534	0.740	3.16	3.20	2.00	2.05
6	35.62	32.12	0.568	0.840	1.28	1.25	1.12	1.00
7	10.00	9.80	0.348	0.460	0.92	0.80	0.43	0.41
8	29.80	30.50	0.224	trace	1.36	1.22	0.95	0.67
9	34.20	38.40	n.d.	n.d.	1.28	1.48	0.285	0.32
10	8.00	8.10	1.20	1.20	0.49	0.32	0.064	trace
11	15.80	14.80	16.28	18.50	1.92	2.50	0.870	0.610
12	8.60	11.50	15.68	20.00	2.24	2.50	0.032	trace
13	3.80	5.20	0.580	trace	0.92	0.80	0.016	n.d.
14	12.40	14.00	10.30	11.00	1.64	1.63	0.076	trace
15	14.60	15.20	15.50	13.00	2.90	1.87	0.042	trace
16	16.00	13.70	25.50	25.00	1.88	1.50	0.180	0.130
17	8.50	8.80	14.00	13.00	1.40	1.25	0.030	0.046
18	13.00	13.20	17.50	14.40	1.08	1.12	0.050	0.042
19	4.77	4.84	5.80	5.00	0.42	0.37	5.57	
20	n.d.	n.d.	0.014	n.d.	0.004	n.d.	n.d.	n.d.
21	0.165	0.120	0.115	0.105	0.002	n.d.	n.d.	n.d.
22	0.093	0.101	0.140	0.117	trace	n.d.	n.d.	n.d.
23	0.042	0.074	0.029	trace	trace	n.d.	n.d.	n.d.
24	0.240	0.220	0.113	0.130	0.0005	n.d.	n.d.	n.d.
25	0.042	0.078	0.055	0.070	n.d.	n.d.	n.d.	n.d.
standard	19.2		11.20		4.2.		0.35	0.37

n.d. = not detectable
1 - 4 = sera of healthy sheep
5 -10 = sera of lambs, 1 - 5 days old
11 = serum of sheep with pulmonary adenomatosis
12 = serum of sheep with endometritis and endocarditis
13-18 = serum-samples taken of a sheep infected intrapulmonary with maedi/visna
19 = lung fluid of No. 11
20 = CSF of No. 11
21 = CSF of healthy sheep
22-25 = CSF of healthy sheep nos. 1 - 4

In Table 1, the results of the determinations of immunoglobulin (sub)class concentrations in mg/ml. of sera, lung fluid and CSF sample of the sheep described under Materials and Methods, are listed.

The relationship between immunoglobulin (sub)class concentrations in ovine body fluids as determined by the nephelometric method and

radial immunodiffusion is shown in scatter diagrams (Figs. 3 a-d).

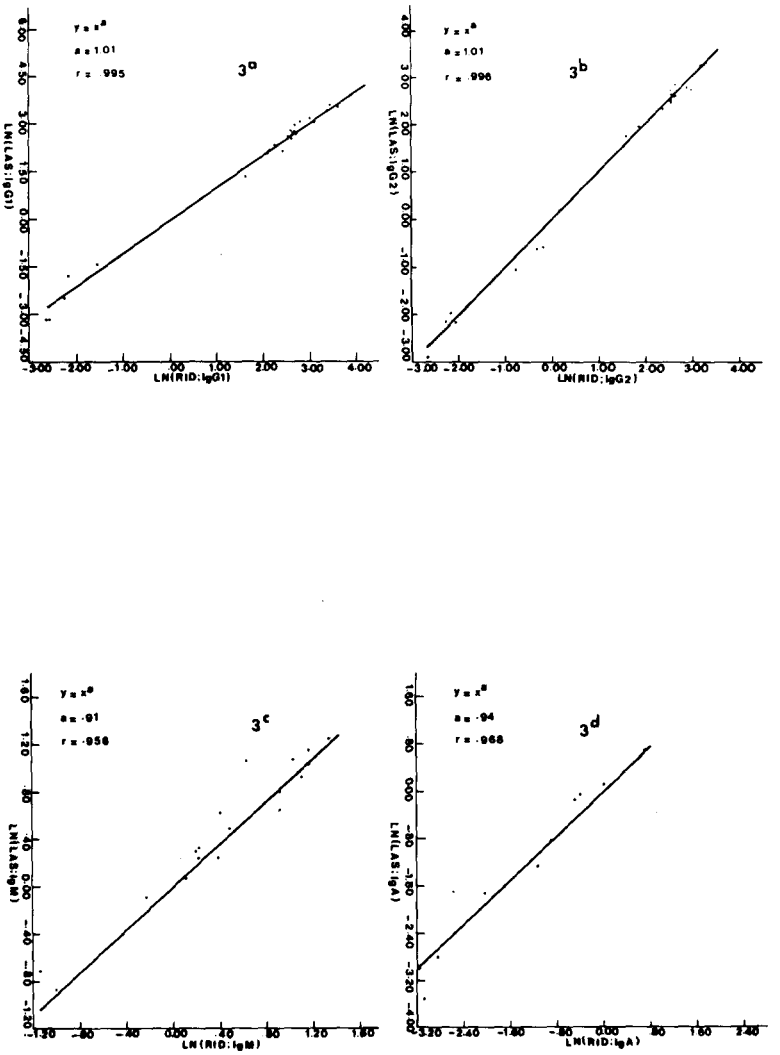

Fig. 3. Scatter diagrams of IgG1 (3a), IgG2 (3b), IgM (3c) and IgA (3d) levels in ovine body fluids in which a laser nephelometer system (LAS) is compared with single radial immunodiffusion (RID).

TABLE 2

Estimation of coefficient a in LN (LAS) = a x LN (RID).

Comparison of RID with LAS assay for measuring	a	σ(a)	r	P	σ(e)	(LAS)
IgG1	1.01	.0175	.995	.59	.23	.16
IgG2	1.01	.0183	.996	.67	.18	.13
IgM	.91	.0515	.956	.10	.18	.13
IgA	.94	.0543	.968	.28	.35	.25

a = estimated value of coefficient a
σ(a) = standard error of a
r = coefficient of determination
P = probability under the hypothesis E (a) = 1
σ(e) = standard deviation of residuals
(LAS) = estimated standard deviation of LAS residuals

Table 2 shows the estimated value of coefficient a for determination of each immunoglobulin (sub)class, its error (= σ(a)), the coefficient of determination (r), the probability under the hypothesis E (a) = 1 (= P), the standard deviation of the residual (=σ(e)) and the estimated standard deviation of the LAS residuals (= σ(LAS)). (LAS) was calculated as the root of the difference between σ^2 (e) and the day-to-day variance of (RID) calculated from Table 3: σ^2 (e) - σ^2 (RID).

Preliminary precision data was calculated using a limited number of runs. The assays in radial immunodiffusion as well as those in nephelometry were carried out for measurement of IgG1.

The results are listed in Table 3. Sera from old sheep as well as from young lambs and one sample of lung fluid and CSF were used in this assay. Variance analysis of LN of the values has been carried out, the relative error was found to be 6.9 per cent in assays with the laser nephelometer and 7.3 per cent in assays with the single radial immunodiffusion test.

TABLE 3

Day-to-day precision for radial immunodiffusion (RID) and laser nephelometric (LAS) assay of IgG1 in ovine body fluids. Sample nos. correspond with nos. in Table 1.

Sample	LAS mean; mg/ml	CV %	RID mean; mg/ml	CV %
1	18.82	3.1	15.08	4.8
2	20.97	7.6	16.93	7.4
3	19.97	4.5	22.83	4.9
4	23.72	1.5	21.05	10.9
5	34.40	4.2	37.95	1.7
6	35.62	7.4	32.12	8.2
19	4.77	9.6	4.84	3.5
22	0.093	10.7	0.101	9.9

DISCUSSION

Studies on the IgG1 and IgG2 subclasses and their respective concentrations in sheep are limited (e.g. Nansen and Aalund, 1972; Conde et al., 1975; Verdouw-Chamalaun et al., 1977; Ciupercescu, 1977), although it is known (Bokhout, 1975 a,b) that adequate reproducibility is only attainable in radial immunodiffusion when IgG titers are determined with subclass-specific antisera. So, although determination of the concentrations of IgG subclasses is unusual in human immunology, even in nephelometric assays, it was decided to use subclass-specific antisera both in these assays and in determinations by RID.

As stated in the introduction, definite conclusions should not be drawn from the absolute values because of the small number of determinations in each group. Nevertheless, the concentrations of Ig (sub)classes determined in normal sheep and young lambs did not differ from the findings previously reported (Verdouw-Chamalaun et al., 1977). Smith et al. (1975) recorded levels of the total IgG in the serum of fifty adult sheep, which were lower than those in the present study when the levels of IgG1 and IgG2 were added. This could be due to the fact that when more than one subclass of the same immunoglobulin is present in a mixture of antigen, the presence of one or several subclass-specific antibodies will result in the production of precipitation rings in RID, which are smaller than those produced by comparable monospecific antisera (Bokhout, 1975b).

Our data, including the very low concentrations of IgG2 in the sera of newborn lambs, are in accordance with those recorded by

Ciupercescu (1977), although this author found lower IgG2 levels in adult sheep. It may be possible that differences in breed account for this.

Smith et al. (1975) were the first authors to report high concentrations of IgA in lung fluid collected in clinical cases of pulmonary adenomatosis (jaagsiekte). They therefore even suggested that this fluid might serve as starting material for the preparation of sheep IgA. In our case of pulmonary adenomatosis the IgA concentration in the lung fluid was also very high, while its serum IgA was about ten times as high as that in sera of normal adult animals. Unfortunately, this finding cannot be compared with levels reported in literature as neither Smith et al. (1975) nor Watson and Lascelles (1971) refer to any concurrent study of the serum and lung fluid of sheep with pulmonary adenomatosis. The findings suggest an overflow of IgA from the lungs into the circulation of these cases. The levels of the IgG subclasses of this particular case of pulmonary adenomatosis were not increased.

In human clinical immunology, considerable attention is paid to examination of the cerebrospinal fluid, not only to detect any mechanical obstructions but also for the diagnosis of particular diseases (such as multiple sclerosis) which are associated with local immunoglobulin production. The protein constituents of normal CSF may be described as being largely similar to the serum protein patterns, although the absolute concentrations are lower. The presence of almost all serum proteins has been clearly demonstrated in human CSF, although IgM was never found to be present in normal CSF. In cases of trypanosomiasis, neurosyphilis and multiple sclerosis, however, IgM is detectable in the CSF. (Schulze and Heremans, 1969).

To our knowledge, the immunoglobulin levels recorded in nos. 21 - 25 (Table 1) are the first CSF levels observed in sheep. A striking feature was that trace amounts of IgM were detectable in the CSF of nearly all healthy sheep. IgA was not detected in any of these cases.

It is commonly believed that the bulk of CSF proteins is derived from plasma by a process of molecular sieving. The concentration of IgA in sheep serum obviously is too low to pass through the basement membrane of the choroid plexuses in measurable amounts. Diseases of the central nervous system (e.g. visna) also occur in sheep; it would be very interesting to examine the CSF for locally produced immunoglobulins in these and similar diseases.
As regards the actual comparison of estimations of the concentrations

of immunoglobulins by laser nephelometry and radial immunodiffusion, Table 1 and statistical analyses (Fig. 3, Table 2) show that the LAS = RID model is a highly satisfactory one, i.e. using laser nephelometry as a substitute for radial immunodiffusion satisfies all practical requirements. When the second-(a_2) and third-degree (a_3) coefficients for the determination of IgG1 by the two methods are ignored, r decreases from .997 to .995; when the zero-(a_o) degree term for the determination of IgM is ignored, r is merely reduced from .969 to .956. Besides in regard to the final results in this sheep model, the nephelometry and RID methods yielded comparable results.

Reproducability was the same in our testing systems, while the reproducibility of the levels recorded with the laser nephelometer were comparable to these attained with commercial anti-human immunoglobulin antisera (ranging from 5 to 8 per cent, Deaton et al. 1976). This confirmed that the nephelometric assay which is commercially available for human clinical studies, may be used in determining animal immunoglobulins, provided suitable antisera can be obtained.

The fact should be stressed that, on the whole, determination of immunoglobulins by laser nephelometry can be more accurate and reproducible. In addition to errors in pipetting, lack of homogeneity of the plates and errors in reading rasults, the diffusion times of the various immunoglobulins are a factor in RID.

An obvious advantage of laser nephelometry is that the results can be known within a few hours. When the immunoglobulin levels are beyond the range of measurement, other dilutions can be immediately used. The measuring range of RID is only adequate from 4.5 to 7.5 mm.; when, for instance, the IgM levels are found to be beyond this range within 60-80 hours, the exact concentrations of immunoglobulin will not be known until seven days later when the study has to be repeated.

Serious errors are associated with the use of the RID method on concentrated CSF and the higher sensitivity of the nephelometric assay makes it the method of choice for determining immunoglobulins in CSF. The increase in sensitivity arises from the fact, that 100 µl. may be used in the nephelometric assay whereas only up to 5 µl. may be used in RID.

CONCLUSION

It can be concluded that, provided the antisera are treated correctly, the use of laser nephelometry in determining concentrations

of immunoglobulins in ovine body fluids not only provided an adequate alternative to radial immunodiffusion but also offers obvious advantages in particular respects.

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