

**Brief Communication**

**INDIRECT IMMUNOFLUORESCENCE USING F(ab')<sub>2</sub>-IMMUNO-REAGENTS FOR THE DEMONSTRATION OF BOVINE VIRAL DIARRHEA VIRUS (BVDV) ANTIGEN IN LYMPHOID TISSUE**

The immunofluorescence technique has proven a valuable tool in the investigation of viral replication *in vivo* and *in vitro*. In our studies on the pathogenesis of BVD special attention was focused on antigen localization in lymphoid tissue and on the characteristics of virus replication in bovine macrophages. In this connection it was considered momentous to be able to differentiate between possible membrane-associated and cytoplasmatically located viral antigens. This led to considerations on possible non-specific binding of conventional immunological reagents to Fc receptors on the cells. Receptors for the Fc portion of IgG have been described on bovine lymphocytes, polymorphonuclear neutrophils and macrophages (Rossi & Kiesel 1977, Grewal *et al.* 1978, Rothlein *et al.* 1980). Moreover, receptors for IgG have been demonstrated in vascular endothelia of cattle (Kasukawa *et al.* 1980).

It has been claimed that surface membranes are destroyed by normal fixation methods (Hijmans *et al.* 1969), but Biberfeld *et al.* (1974) showed that, although damage did occur during acetone fixation, it was not complete. Acetone made cells partly permeable to immunoglobulin, facilitating intracellular penetration of FITC-conjugates, but left some cell surface antigens intact as revealed by incomplete elimination of specific staining of surface-bound immunoglobulins. We assumed that some Fc receptors might also pass this treatment undamaged, especially when 20 % acetone solution was used for fixation to obtain a better preservation of morphology. We therefore concentrated on the use of antibodies where the Fc portion was removed by pepsin treatment. Initially, direct immunofluorescence using FITC-conjugated F(ab')<sub>2</sub>-fraction of bovine anti BVDV hyper-immune serum was tested, but the intensity was too low for practical use. The & Feltkamp (1970) have shown that a direct correlation between the binding affinity in immunofluorescence and the precipitating activity of antibodies does exist. In contrast to porcine immunoglobulins, bovine immunoglobulins possess relatively poor precipitating activity (Dalsgaard, unpublished observation), and the activity may be further reduced after pep-

sin treatment in the production of  $F(ab')_2$ -fragments (Møller 1979).

Amplification of the system was finally achieved by the indirect method using FITC-conjugated  $F(ab')_2$  anti species reagent, and by the application of  $F(ab')_2$ -fragments from porcine hyperimmune serum against BVDV:

Hyperimmune antiserum against BVDV was raised in pigs by repeated intravenous injection of the Danish strain Ug 59 (Ohmann et al., in prep.).

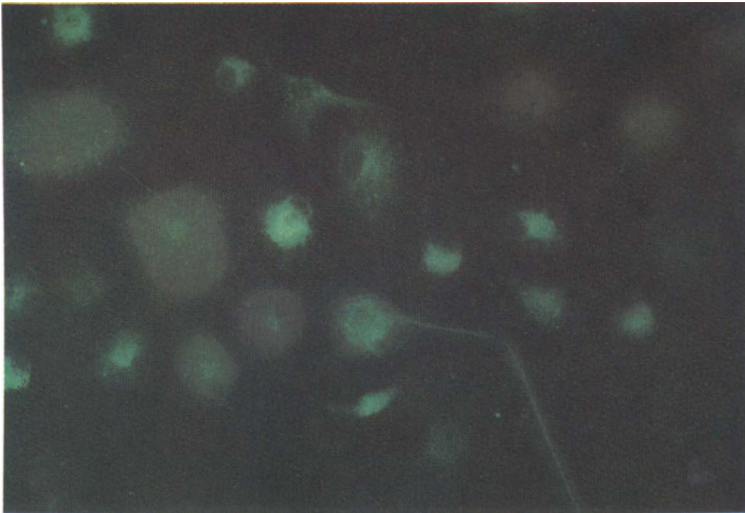
Rabbit antiserum against swine IgG was prepared essentially by the method of Harboe & Ingild (1973).

$F(ab')_2$ -fragments of swine and rabbit serum were prepared according to Poulsen & Hjort (1980) using pepsin digestion of total serum protein. An enzyme protein ratio of 1:50 was used overnight at pH 4/37°C. After centrifugation and dialysis the fragments were purified by gel chromatography on Ultrogel ACA 34 (LKB products). By SDS-polyacrylamide gel electrophoresis (Laemmli 1970) it was confirmed that the method yielded relatively pure  $F(ab')_2$ -fragments. The elimination of Fc-mediated complement fixing activity was confirmed by the method described by Eskildsen (1975).

FITC-conjugation of rabbit  $F(ab')_2$  was performed by the method described for total IgG by The & Feltkamp. By the biuret method (Kabat & Mayer 1964) the extinction  $E_{1\text{ cm}}^{280}$  of a 1% solution of  $F(ab')_2$  was estimated at 8 and of intact IgG at 12.  $F(ab')_2$ -conjugation products with an FITC/Protein ratio about 50% higher than described for intact IgG were used accordingly. Fluorescein isothiocyanate (FITC) isomer 1 (Baltimore Biological Laboratories) was used for the conjugation. The conjugates were stored in the dark at 4°C with 0.2 mg/ml  $\text{NaN}_3$  added.

Bovine macrophage cultures, unfixed or fixed in 20% buffered acetone, and appropriate cryostat sections, prepared as described elsewhere (Ohmann et al.), were stained by incubation for 60 min with  $F(ab')_2$  swine anti BVDV (diluted 1:80) followed by 4 washings in PBS, 5 min each, and by 30 min incubation with the FITC-conjugate (diluted 1:40). Reactions were run in moist chambers at 37°C. Three washings in PBS and 1 in distilled water removed unreacted conjugate. Fluorescence, specific for BVDV antigen, was confined to the cell cytoplasm, often with highest intensity and a granular distribution in the juxta-nuclear region. In tissue sections, the specific reaction was easily distinguished from occasional yellowish auto-fluorescing

*Helle Bielefeldt Ohmann & Kristian Dalsgaard: Bovine viral diarrhoea virus.*



**Figure 1.** Bovine macrophage culture 8 days after inoculation of BVDV. Staining with swine  $F(ab')_2$  anti BVDV followed by FITC conjugated rabbit  $F(ab')_2$  anti porcine IgG.

clumps, related to cell debris or to granulocytes. The non-specific staining of elastic and collagenic components seen in preparations stained by the application of whole bovine antisera was negligible.

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#### REFERENCES

- Biberfeld, P., G. Biberfeld, Z. Molnar & A. Fagraeus*: Fixation of cell-bound antibody in the membrane immunofluorescence test. *J. Immun. Meth.* 1974, 4, 135—148.
- Eskildsen, M.*: Complement fixation with porcine serum antibodies. *Acta path. microbiol. scand. Sect. C* 1975, 83, 315—324.
- Grewal, A. S., B. T. Rouse & L. A. Babiuk*: Characterization of surface receptors on bovine leukocytes. *Int. Arch. Allergy* 1978, 56, 289—300.
- Harboe, N. & A. Ingild*: Immunization, isolation of immunoglobulins, estimation of antibody titre. *Scand. J. Immunol.* 1973, 2, Suppl. 1, 161—164.
- Hijmans, W., H. R. E. Schuit & F. Klein*: An immunofluorescence procedure for the detection of intracellular immunoglobulins. *Clin. exp. Immunol.* 1969, 4, 457—472.
- Kabat, E. A. & M. M. Mayer*: *Experimental Immunochemistry*. 2nd Ed., Thomas, Springfield, Illinois 1964, 559—563.
- Kasukawa, R., M. Okada & S. Igari*: Receptors for IgG Fc and complement in the mammalian aorta. *Int. Arch. Allergy* 1980, 61, 175—182.
- Laemmli, U. K.*: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 1970, 227, 680—685.
- Møller, N. P. H.*: Fc-mediated immune precipitation. I. A new role of the Fc-portion of IgG. *Immunology* 1979, 38, 631—640.
- Ohmann, H. B., M. Holm Jensen, K. J. Sørensen & K. Dalsgaard*: Demonstration of bovine viral diarrhoea antigen in tissue sections by immunoperoxidase assay. In preparation.
- Poulsen, F. & T. Hjorth*: A simple method for the production of F(ab')<sub>2</sub> preparations by pepsin digestion of total serum protein. *Acta path. microbiol. scand. Sect. C* 1980, 88, 241—245.
- Rossi, C. R. & G. K. Kiesel*: Bovine immunoglobulin G subclass receptor sites on bovine macrophages. *Amer. J. vet. Res.* 1977, 38, 1023—1025.
- Rothlein, R., D. W. Johnson & C. C. Muscoplatt*: Enumeration and characterization of bovine blood, spleen and lymph node cells containing immunoglobulins. *Int. Arch. Allergy* 1980, 61, 100—106.
- The, T. H. & T. E. W. Feltkamp*: Conjugation of fluorescein isothiocyanate to antibodies. I. Experiments on the conditions of conjugation. *Immunology* 1970, 18, 865—873.

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