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Canine parvovirus: development of immunofluorescence and immunoperoxidase techniques

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Two methods of immunocytochemistry, immunofluorescence (IFA) and immunoperoxidase (PAP) were used to demonstrate canine parvovirus (CPV) antigens in sections of canine tissue. Specific staining using IFA and PAP was successful only in sections of fresh frozen tissue and formalin fixed/formol sublimate postfixed tissues respectively. A range of tissues was then taken at post mortem examination from a puppy which had been experimentally infected with CPV. Upon comparison, PAP staining gave high resolution, a permanent preparation and clear intracellular localisation of antigen. IFA resulted in less defined localisation of antigen but the technique was simpler and more easily controlled.

CANINE parvovirus (CPV) has been associated with two distinct disease syndromes; myocarditis as a result of perinatal infection and enteritis following infection of older dogs (McCandlish et al 1981).

The pathogenesis of CPV enteritis has been defined (Macartney et al 1984a,b) using immunofluorescence and immunoperoxidase. The present report describes the earlier development of these methods, together with a critical comparison of their use.

Immunofluorescence (IFA) has been widely applied in the study of infectious disease. In the dog, it has been used in the diagnosis and experimental study of canine adenovirus infection (Wright and Burns 1966) and canine distemper virus infection (Fairchild et al 1967, Appel 1970). More recently, Keenan et al (1976) applied IFA to the study of canine coronavirus infection.

However the inherent difficulties of IFA — the use of frozen sections to minimise loss of antigenicity, the requirement for incident light fluorescent microscopy and the impermanence of the preparation — have led to the development of other, non-fluorescent, immunocytochemical methods (Sternberger 1974). By far the most widely applied are those using horseradish peroxidase.

Immunoperoxidase methods have been reviewed by Heyderman (1979); direct and indirect methods, using conjugated enzyme, are available. However, these have been largely superseded by the peroxidase/ anti-peroxidase (PAP) technique developed by Sternberger et al (1970). In this method maximal immunological reactivity is maintained as damaging chemical conjugation procedures are not used. Instead, the enzyme is coupled to the antibody system immunologically. This permits the use of a higher dilution of primary antiserum and, since only small numbers of antigenic sites are required, the method may be applied to fixed and processed tissues. The PAP technique has been applied in veterinary research; Cartew (1978) used the method to study mouse viral hepatitis, while Ducatelle et al (1980) applied it to canine distemper virus infection.

In the present report PAP was used in conjunction with IFA and the relative merits of the techniques were assessed.

Materials and methods

Production of antisera

Six young healthy rabbits were selected. Sixty millilitres of blood was removed from each rabbit and the separated serum subsequently used as a negative control for each specific antiserum produced. The rabbits were then inoculated intramuscularly with 1 ml of a 50:50 emulsion of CPV and Freund’s complete adjuvant. The virus was produced by purification from faecal material as described by Macartney et al (1984a). Six weeks after this primary immunisation, each rabbit was inoculated intravenously with a smaller dose of virus. All animals were exsanguinated six days following intravenous inoculation and the serum removed. Titres of antisera were assessed using a hamagglutination inhibition test as described by Macartney et al (1984a) and are shown in Table 1. The serum of rabbit 3, with the highest titre, was used in the present study.

Infected tissues

For the initial developmental work it was decided to use sections of myocardial tissue from pups with CPV myocarditis. This tissue was selected since it provided
FIG 1a: Control myocardium. A large darkly basophilic bar-shaped inclusion body is apparent within an affected cardiac myocyte (arrow). Haematoxylin and eosin × 250

FIG 1b: Control myocardium. A single brightly fluorescent nucleus is apparent within an affected cardiac myocyte (arrow). There is little background staining. IFA × 250

FIG 1c: Control myocardium. A single darkly stained nuclear inclusion body is apparent within an affected cardiac myocyte (arrow). Definition is better than with IFA and there is little background staining. PAP × 250
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TABLE 1: Titres of specific antisera to CPV (HI)

| Rabbit number | HI titre* |
|---------------|-----------|
| 1             | 65,000    |
| 2             | 8192      |
| 3             | 1,040,000 |
| 4             | 32,768    |
| 5             | 32,768    |
| 6             | 130,000   |

* Results expressed as the reciprocal of the highest dilution completely inhibiting haemagglutination

Histological procedures

The formalin fixed myocardial tissue blocks were divided and a portion was post fixed in formal sublimate for 24 hours. This block, its duplicate formalin fixed block and the Bouin’s fixative, ethanol, methanol and paraformaldehyde/glutaraldehyde. In addition blocks were snap frozen in liquid nitrogen. Light microscopical examination of formalin fixed material revealed large numbers of basophilic intranuclear inclusion bodies in cardiac myocytes (Fig 1a). Ultrastructural examination revealed that the inclusions were composed of aggregates of round particles 20 nm in diameter, morphologically identical to CPV virions (Fig 2).

To compare the quality of staining using IFA and PAP, tissues were taken from an experimentally infected puppy five days after oral inoculation with CPV. At post mortem examination duplicate blocks of the following tissues were taken and fixed either in NBF/formol sublimate or snap frozen in liquid nitrogen: submandibular, retropharyngeal, mesenteric and popliteal lymph nodes, thymus, palatine tonsill, spleen, lung, heart, liver, kidney, bone marrow, stomach, duodenum, jejunum, ileum, caecum and colon. With the exception of stomach and caecum all alimentary tissues were pinned flat on cork blocks before fixation.

Immunofluorescence

Initially both frozen and paraffin embedded tissues were used. Two sections were cut from each tissue block; the sections from wax embedded blocks were dewaxed and rehydrated. One section was incubated with the specific antiserum and the other with the preimmunisation serum from the same animal. Following incubation at room temperature for 30 minutes they were washed in three changes of phosphate buffered saline (PBS) pH 7.2, under constant agitation. All sections were then incubated with fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG (Sigma) again for 30 minutes at room temperature, washed as before and mounted in 50 per cent glycerol in PBS.

Peroxidase/antiperoxidase (PAP)

The immunoperoxidase technique was performed as shown diagramatically in Fig 3. Following hydration and removal of mercuric pigment where necessary, sections were incubated with 0.5 per cent hydrogen peroxide in methanol for 30 minutes at room temperature and washed twice, initially in Tris buffer pH 7.6 and secondly in Tris solution containing 1 per cent normal swine serum. The sections were then incubated with serial dilutions of the primary antiserum in PBS pH 7.8 with 0.1 per cent bovine serum albumin and 0.1 per cent sodium azide, for 18 hours at 4°C. The washing steps were repeated and a commercial antiserum to rabbit IgG, raised in swine, was layered on to the sections and incubated for one hour at 20°C. The washing steps were again repeated and the sections incubated with peroxidase/anti-peroxidase raised in rabbit, for one hour at 20°C. The sections were then washed and immersed in a solution of 0.05 per cent diaminobenzidine in Tris buffer with 3 per cent hydrogen peroxide, for five minutes at room temperature. Following this enzymatic reaction, the sections were washed in tap water, counterstained with haematoxylin and mounted routinely.

For each section subjected to the entire staining procedure, three control sections were also examined. Control 1 was subjected to the entire staining procedure, except that the preimmunisation serum was substituted for the specific antiserum. In control 2, the specific antiserum and the bridging antibody were omitted. This was to ensure there was no cross-reactivity between the PAP complex and the section. Control 3 was incubated only with the diaminobenzidine solution to ensure elimination of endogenous peroxidase activity in the section.
Initially, two sections from fixed tissues were stained. One section was incubated with trypsin solution before incubation with the specific antiserum and the remaining section left untrypsinised. Trypsin digestion was carried out at 37°C for 30 minutes using freshly prepared 1 per cent trypsin solution in Tris buffer pH 7.8 with 0.01 per cent calcium chloride. Brown stain precipitate, was apparent over the nuclei of affected cardiac myocytes (Fig 1c). Staining was well localised with little background staining.

Optimal dilutions of the antiserum were determined as 1/100 and 1/1000 for IFA and PAP respectively. Antiserum was used at these dilutions for all further studies.

**Results**

*Staining of control myocardial tissue*

The results are shown in Table 2.

Specific fluorescence was apparent only in frozen tissues. Staining was localised to nuclei of infected cells with little background staining (Fig 1b).

In contrast, PAP staining was successful only in NBF/sublimate fixed specimens which had been trypsin digested. Staining, denoted by an insoluble brown stain precipitate, was apparent over the nuclei of affected cardiac myocytes (Fig 1c). Staining was well localised with little background staining.

Optimal dilutions of the antiserum were determined as 1/100 and 1/1000 for IFA and PAP respectively. Antiserum was used at these dilutions for all further studies.

**TABLE 2: Results of staining control myocardial tissue**

| Fixative         | IFA | PAP with predigestion | PAP without predigestion |
|------------------|-----|-----------------------|--------------------------|
| 10% NBF          |     |                       |                          |
| 10% NBF/sublimate|     | +                     |                          |
| Bouin's fixative |     |                       |                          |
| Methanol         |     |                       |                          |
| Ethanol          |     |                       |                          |
| Fresh frozen     | +   | ND                    |                          |

ND Not done
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rabbit-anti-
peroxidase

swine-anti-
rabbit

rabbit-anti-
CPV

DAB

5 min room temp

60 min room temp

18 h 4°C pH 7.8
with 0.1% BSA

1% trypsin pH 7.8
with 0.01% CaCl₂

FIG 3: Diagrammatic representation of finalised method of PAP technique. DAB Diaminobenzidine, BSA Bovine serum albumin, hpr Horseradish peroxidase

Staining of experimentally infected tissue

In view of the results presented in Table 2, PAP and IFA staining was performed on NBF/sublimate fixed and frozen sections respectively.

A similar distribution of viral antigen in the tissues was detected using both IFA and PAP methods. Specific staining was present in the thymic cortex, lymph nodes, spleen and intestine. In the thymus, staining was weak and antigen was uniformly extranuclear. Using IFA this antigen appeared as a fine granular deposit, outlining individual thymocytes. However PAP staining resulted in much more accurate localisation of viral antigen, with clearly defined linear stain precipitates lying between lymphoid cells.

Antigen was present in the germinal centres of the lymph nodes and gut associated lymphoid tissue. In contrast to the thymus, antigen was present both within the nuclei of lymphocytes and the intercellular ground substance. Using IFA the intranuclear antigen was well defined; however extranuclear staining was observed as a fine granular deposit (Fig 4a). It was not possible to ascertain the exact location of this antigen. PAP on the other hand, while demonstrating nuclear staining with equal efficiency, was able to localise extranuclear antigen more accurately (Fig 4b). This antigen was present in the extracellular ground substance and did not appear to be within the cytoplasm of histiocytic reticulum cells.

Specific staining was apparent throughout the small intestine. In the duodenum, staining was predominantly extracellular. While PAP staining resulted in more accurate localisation of antigen, there was considerable non-specific background staining. Less background staining was apparent using IFA, although extracellular staining in the lumina of crypts was more difficult to interpret. Intranuclear antigen was well defined using both methods. Using PAP, this staining was associated with intranuclear inclusion bodies. In the jejunum and ileum both techniques accurately localised antigen to individual cells in the proliferative crypt epithelium (Figs 5a and b). Further, PAP revealed that this antigen was within the nuclei of small darkly pyknotic cells which had been observed on routine histological sections (Macartney et al 1984a). A few positively staining cells were present in the crypts of the caecum and colon. Staining was again intranuclear.

Viral antigen was also detected, using both techniques, in the bone marrow and in the liver. In the marrow, antigen was present within the nuclei of medium sized cells. It was difficult to identify accurately these cells, even using PAP, since the brown stain precipitate tended to overlie and obscure nuclear morphology. In the liver, antigen was present within the cytoplasm of Von Kupffer cells (Fig 6). IFA permitted accurate localisation of antigen with little background staining. In contrast, there was extensive non-specific background staining using PAP.

Discussion

The choice of myocardial tissue for developing immunocytochemical techniques proved to be justified. This tissue provided a well localised site of viral antigen with minimum background staining.

Immunofluorescent staining was successful using only frozen tissue sections presumably because of complete preservation of antigenic reactivity in the section. The failure to demonstrate antigen in the tissues fixed in NBF and Bouin’s fixative was almost certainly due to destruction of antigenic sites in the section by these aldehyde fixatives. It is possible that enzymatic predigestion, similar to that applied in the PAP technique, may have exposed antigenic sites and
FIG 4a: Lymph node. Nuclear antigen is well defined (large arrow). However extranuclear antigen is apparent as a poorly localised granular deposit (small arrow). IFA × 400

FIG 4b: Lymph node. Using PAP, both intranuclear (large arrow) and extranuclear antigen (small arrow) are well defined. PAP × 250
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allowed specific immunofluorescent staining. It is disappointing that there was no specific staining following ethanol or methanol fixation, as this type of fixative has been used successfully in the staining of immunoglobulin and feline leukaemia virus antigens in tissue sections (Sainte-Marie 1962, Dorsett and Joachim 1978). The reason for the failure of these alcoholic agents to preserve antigenicity is unknown. The denaturation of precipitated proteins upon rehydration of the tissue (Sternberger 1974) may, in part, have been responsible. Since covalent or hydrophobic bonds are not involved in precipitation by these fixatives, enzymatic pretreatment of alcohol fixed tissue would be inappropriate. However it may be that alterations in the concentration of fixative and the duration of fixation would have resulted in a balance between morphological preservation and antigenic reactivity.

The PAP technique has three outstanding features. The first is its permanence; sections may be prepared, examined and stored for retrospective study. Secondly, good histological definition is possible since formalin fixed material may be used. The first two features depend on the third, the sensitivity of this technique, which enables recognition, in fixed tissues, of antigenic sites which have been largely denatured. Paradoxically, it was probably the sensitivity of the PAP technique which led to its failure to stain the intranuclear inclusions in fresh frozen tissues. This, almost certainly, was caused by tight packing of specific anti-CPV antibodies because the minimal fixation procedures would have resulted in the preservation of large numbers of antigenic sites. This high density of primary antibody can lead to loss of the essential bifunctional reactivity of the bridging antibody.

Formalin fixation, by denaturation of the antigenic
sites, leaves sparsely distributed antigen resulting in less dense packing of the primary antibody and a return to the bifunctional reactivity of the bridging antibody. In the present system trypsin digestion of the sections is essential for specific staining. It would appear, therefore, that formalin fixation results in extensive denaturation of the antigenic determinants of CPV. The success of trypsin digestion would suggest that loss of antigenic reactivity is primarily the result of aldehyde fixation and that the effects of heat and organic solvents during processing were likely to be minimal. The failure to stain tissue which had either been fixed in NBF alone or in Bouin’s fixative was again probably the result of aldehyde fixation. Attempts to digest sections of this type with trypsin were unsuccessful, largely due to extensive digestion of the section. Serial reductions in length of trypsinisation have subsequently been attempted but it has not been possible to define a balance between digestion of the section and antigenic reactivity with these fixatives. The hardening effect of mercuric chloride in the sublimate solution would therefore appear to be essential for successful staining.

The success of PAP using formalin fixed, sublimate post fixed tissue was remarkable. This fixation regime is widely used in routine processing. It is therefore possible, using the PAP technique, to perform retrospective studies on stored, paraffin embedded, tissue blocks.

The distribution of antigen using both IFA and PAP correlated with the severity and extent of histological lesions in the lymphoid and alimentary tissues (Macartney et al 1984a). However upon comparison of the two methods, the advantages of each were apparent. Immunofluorescence yielded an impermanent preparation and, although intracellular antigen was well defined, extracellular staining was more difficult to interpret. The advantage of the technique was its simplicity, requiring only one control section for every one stained. This, together with its relatively low demand on time, would commend its use as a rapid ‘screening’ technique for large numbers of tissues.

The PAP method, on the other hand, gave a permanent preparation in which there was greater definition. It was possible accurately to associate antigen with specific cell types noted on histological examination. Extracellular antigen was more accurately localised and defined than with immunofluorescence. However, background staining was a problem, particularly in the intestine, and could have led to difficulty in interpretation of preliminary findings. This background staining was probably caused by non-specific binding of the bridging antibody to precipitated plasma proteins in the severely damaged intestinal tissues. PAP was also more complex technically and demanding on time. These latter features would militate against its use for screening large numbers of tissues, especially if frozen material was available. However, its use in accurately localising antigens to high resolution makes it invaluable for detailed studies.

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