Group-specific and Type-specific Gel Diffusion Precipitin Tests for Bluetongue Virus Serotype 20 and Related Viruses

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Abstract

Using antigens prepared from cell cultures infected by bluetongue (BLU) virus type 20 (BLU-20), and sera from cattle which had recovered from experimental infection by that virus, two distinct precipitin reactions were demonstrated by immunodiffusion. Two distinct gel diffusion precipitin tests were developed based on these reactions. The antigen of one was common to BLU-20 and two other Australian BLU isolates, CSIRO 154 (BLU-21) and CSIRO 156 (BLU-1). It was therefore concluded to be a group-specific test. The antigen of the second appeared to be unique to BLU-20. The test based on this antigen correlated well with the virus neutralization test for BLU-20 and it was therefore concluded to be type-specific.

Similar methods applied to a virus of the Palyam (PAL) group demonstrated two precipitin reactions of similar broad (group) and narrow (type) specificity.

Introduction

Following the isolation of BLU-20 from *Culicoides* spp. from northern Australia (St George *et al.* 1978) it was necessary to determine the limits of its distribution in the Australian cattle and sheep populations. For various practical reasons it was not feasible to use the type-specific virus neutralization (VN) test in all circumstances or on the required scale.

Gel diffusion precipitin (GDP) tests for BLU have been described by Klontz *et al.* (1963), Jochim and Chow (1969) and Jochim (1976). In these reports the test was presented as being group-specific and based on a single line, although an antigen of a more complex nature was suggested by one report (Jochim 1976) in which soluble and cell-associated antigens sharing partial identity were described.

Because of quarantine restrictions it was not possible to import exotic viruses or material from tests developed outside of Australia, so it was necessary to generate a GDP test using BLU-20. This report describes the development of that test, its validation as a group-specific test for BLU antibody or antigen, and the development of a second GDP test which is type-specific for BLU-20. Reference materials for the group-specific test have subsequently been distributed to all State and Commonwealth veterinary laboratories in Australia, from which extensive surveys of livestock sera have been conducted (Snowdon and Gee 1978; Coackley *et al.* 1980; Flanagan *et al.* 1982; Della-Porta *et al.* 1983; Burton and Littlejohns 1988; Littlejohns and Burton 1988; Littlejohns *et al.* 1988). Materials have similarly been provided to a number of overseas countries, including Papua New Guinea (Van Kammen and Cybinski 1981), Indonesia (Sendow *et al.* 1986), Uraguay (St George, unpublished data) and elsewhere. The exact origin and nature of the test and the reference materials required for its performance, and considerations regarding its cross reactivities, should qualify all of that usage.

Definitions and Abbreviations Used

BLU, bluetongue. Hyphenated numeral indicates serotype. **DR**, double referenced. Describes a BLU group-specific GDP line or test for which both reference antigen and reference antiserum are of known origin. Compare with SR. **GDP**, gel diffusion precipitin. **PAL**, palyam. **SR**, single referenced. Describes a BLU group-specific GDP line or test for which the exact origin of only the reference antigen is known, the reference antiserum being of field origin and possibly the result of exposures to related viruses. Compare with DR. **TCID$_{50}$**, 50% infectious dose for cell cultures. **VN**, virus neutralization.

Materials and Methods

Viruses

The following viruses were used for antigen production.

BLU-20 (St George *et al.* 1978) was passed up to 10 times in BHK or Vero cells. Other BLU viruses, CSIRO 154 (BLU-21) and CSIRO 156 (BLU-1) (St George *et al.* 1980) were passed three times in BHK cells, plaque purified twice in Vero cells and passed a further four times in BHK cells.

Three PAL group viruses, Bunyip Creek, CSIRO Village and D'Aguilar (Cybinski and St George 1982, Knudson *et al.* 1984) were passed five times in BHK cells. A virus of the PAL group, designated M3, was isolated from a cow on the north coast of New South Wales and subsequently passed up to 10 times in BHK cells. In most of the work reported here it was used to represent the PAL group. Virus M3 was identified with Bunyip Creek virus by VN test but is referred to as a separate entity because serotypic identity on this criterion alone may not necessarily imply total identity of other viral proteins or antigens. This qualification may be important when considering questions of group identities and cross reactivities.

Antisera

Initially, BLU-20 antibody was provided by five sera, from three sheep and two calves which were experimentally infected with BLU-20 and bled 20 or 25 d post-inoculation. Later, a field serum which was found to be strongly group antibody reactive, but lacking in BLU-20 neutralizing antibody (D. H. Cybinski, personal communication), was used as an alternative when only group antibody was required. Experimental or field sera were used for the 'double referenced' (DR) or 'single referenced' (SR) forms of the group test, which are described below, respectively. The type test was, by its nature, necessarily of a DR form only.

M3 antiserum was obtained from the cow from which the virus was isolated, 7 weeks later.

Virus Neutralization Test

VN tests were performed by the method of Cybinski *et al.* (1978).

Antigen Preparation

Two types of antigen were used. They will be referred to as 'supernatant antigen' and 'deposit antigen'.

For supernatant antigen, BHK, Vero or McCoy cells in 100 ml medium in rolling bottles of about 1200 cm^2 surface area were infected with 10^6 TCID$_{50}$ virus. When about 80% of the cell sheet showed cytopathic effect the remaining cells were shaken off the glass and the harvest frozen, thawed and centrifuged at 1500 *g* for 60 min. The clarified culture fluid was concentrated up to 100 times by dialysis against polyethylene glycol, MW 20 000. In later routine preparation $\frac{1}{10}$ volume of cold 1 M Tris buffer, pH 7·6, and 0·2% v/v β-propiolactone were added to the culture fluid to inactivate infectious virus (D. A. McPhee, personal communication) before concentration.

For deposit antigen, the deposit from the centrifuged harvest was resuspended in a minimal volume of culture fluid and frozen and thawed.

GDP Test Procedure

Borate buffered agarose gels were set in 90 mm polystyrene petri dishes, 15 ml per plate. Wells, of 5 mm diam., were cut in hexagonal patterns, each pattern consisting of six outer wells placed 7 mm, centre to centre, from a central well and from each other.

For selection of antigens and antisera capable of producing reactions for reference purposes, candidate sera in peripheral wells were diffused against candidate antigens in the central well. Selected

combinations of antigen and antiserum were then similarly tested at dilutions and those giving optimal reference lines, i.e. the sharpest precipitin lines of adequate extent, were chosen to provide reference lines in the routine test.

For a routine test the antigen was placed in the central well and reference antiserum in three alternate peripheral wells to produce reference lines. Test samples were placed in the intervening peripheral wells. The plates were held in a humidified chamber at 15–25°C and read 1 and 2 days later over oblique illumination. The strength of a reaction, either for antigen or antibody, was determined by comparison with the reference lines and recorded as a numerical value (Littlejohns 1981).

Two GDP tests were developed. One, ultimately determined to be group-specific, was based on the supernatant antigen and was used in two forms, DR (double referenced) and SR (single referenced). Initially, a selected BLU-20 antiserum was used as reference antiserum to give the DR form of the test, in which both reference reactants were of known origin. Later, to accommodate the heavy demand of the time, an adequately reactive field serum was substituted as the reference antiserum to give the SR form of the test, in which only the reference antigen was of known origin, although the identity of the reference line was established by comparison with that of the DR test. Apart from the comparison of the two forms as tests for antibody, all results of BLU group tests reported in his paper were from the use of the DR form. Also, only the DR form was distributed overseas. However, the SR form was subsequently used extensively in the investigations into BLU and related viruses in New South Wales, which are described in accompanying papers (Burton and Littlejohns 1988; Littlejohns and Burton 1988; Littlejohns *et al.* 1988) and elsewhere within Australia.

The second GDP test, ultimately determined to be type-specific, was based on the deposit antigen and a BLU-20 serum which was capable of reacting with it to produce two lines, one of which was identical to that of the group test. The group-specific reaction was eliminated by one of two methods:

1   Absorption of group reactivity from deposit antigen with an antiserum, of NSW field origin, which had group reactivity only (negative to the VN test for BLU-20).

2   Absorption of group reactive antibody from the BLU-20 antiserum with a supernatant antigen which had only group reactivity demonstrable.

GDP Reactions of PAL Group Viruses

M3 antigens were prepared as supernatant or deposit antigens in the same ways as were used for BLU antigens. They were diffused against M3 antiserum to give one or two precipitin lines according to the nature of the antigen.

Sera and Antigens to Evaluate BLU and PAL GDP Tests

To compare the two BLU GDP tests, based on supernatant or deposit antigens, they were used on the following samples. Some of these samples were also examined in the PAL putative group test, that is, the test based on the supernatant antigen.

Materials examined are grouped according to purposes and opportunities that emerged sequentially as the background to the use of the tests evolved, that is, from their relevance to naturally acquired (group A) or experimentally induced (group B) antibody to BLU-20 virus, when that was the only Australian BLU virus recognized, and to their cross reactivity with other BLU (group C) and PAL (group D) viruses. Finally, group GDP positive sera from New South Wales cattle which were BLU-20 VN negative (group E) were examined in the type test to evaluate its specificity in that situation.

Group A.   Naturally occurring BLU-20 antisera, of known BLU-20 VN status, coded in one laboratory and tested in another. Fifty-two bovine sera from the Northern Territory where BLU-20 infection is endemic, 26 of which were BLU-20 VN positive and 26 negative.

Group B.   Sera from experimental BLU-20 infections. Fifty-nine sera, from five sheep and two cattle, included 11 sera collected prior to inoculation, which were BLU-20 VN negative, and 48 collected post-inoculation, of which 14 were VN negative and 34 were positive.

Group C.   Materials relating to other BLU viruses. Deposit antigens from BLU-1 (CSIRO 156) and BLU-21 and antisera against BLU-1 (CSIRO 156) (bovine) and BLU-21 (rabbit).

Group D.   Materials relating to PAL group viruses. Deposit antigens from CSIRO Village, Bunyip Creek and M3 virus-infected cells, and rabbit antisera to the first two and D'Aguilar virus. Also 112 sera representing serial weekly bleeds from four calves which had been sequentially exposed to the three PAL virus serotypes.

Group E. To evaluate the specificity of the type GDP test, 47 bovine sera from the north coast of New South Wales, which gave strong reactions in the BLU GDP tests but were negative in the BLU-20 VN test, were examined in the type test.

To compare DR and SR tests, 108 sera from widely separated areas, and including 31, 62 and 15 from zones of high, intermediate and low group antibody prevalence respectively (Burton and Littlejohns 1988) were examined in each.

Experimental Exposure of Calves to the PAL Group Viruses

Four calves, initially aged 3 months, were exposed sequentially to the three PAL group viruses, M3 (serotype = Bunyip Creek), CSIRO Village and D'Aguilar. Inocula used were culture fluids from infected cell cultures, centrifuged to remove cell debris. Doses of about 10^5 $TCID_{50}$ were given intravenously at *c.* 2-month intervals. The animals were bled immediately prior to inoculation and then weekly until 5 weeks after the final exposure, and sera were tested in VN tests for M3, CSIRO Village and D'Aguilar viruses, and in the PAL group and BLU group and type GDP tests.

Table 1. Results of Bluetongue group and type GDP tests on sera and antigens of Bluetongue and Palyam viruses

Source and nature of sample[A]	Number	BLU group GDP test +	BLU group GDP test −	BLU-20 type GDP test +	BLU-20 type GDP test −
Group A, sera from naturally exposed cattle					
BLU-20 VN + ve	26	22[B]	2	25	1
BLU-20 VN − ve	26	2	24	0	26
Group B, sera from experimentally infected cattle					
Pre-inoc, BLU-20 VN − ve	11	0	11	0	11
Pre-inoc, BLU-20 VN − ve	14	0	14	2	12
Post-inoc, BLU-20 VN − ve	34	34	0	34	0
Group C, relating to other BLU serotypes					
Antigens					
BLU-1[C]		1	0	0	1
BLU-21		1	0	0	1
Antisera					
BLU-1[C]	3	3	0	0	3
BLU-21	3	3	0	0	3
Group D, relating to PAL group viruses					
Antigens					
CSIRO Village		0	1	0	1
Bunyip Creek		0	1	0	1
M3		0	1	0	1
Sera[D]	112	0	112	0	112
Group E, sera from naturally exposed cattle outside BLU-20 area					
BLU-20 VN − ve	47	47	0	0	47

[A] See Materials and Methods for details.
[B] Two inconclusive results are omitted.
[C] Australian isolate CSIRO 156.
[D] Includes 106 sera positive in Palyam group GDP test after serial infection of cattle with up to three viruses of the group.

Results

GDP Test for BLU Based on the Supernatant Antigen

One common precipitin line of varying strength and position occurred when each of the five BLU-20 antisera were diffused against supernatant antigen. One serum gave a line, of

adequate strength and clarity, which formed midway between the antigen and serum wells. Antigen and this serum, when used in concentrations which gave an optimum reference line, were then used as reference reactants to set up a test. Results of the application of this test to various sera and antigens (Table 1) indicate group specificity.

From the 52 sera from the Northern Territory (group A), of 26 positive in the BLU-20 VN test, 22 were positive, 2 doubtful and 2 negative in this GDP test. Of the 26 sera which were negative in the VN test, 2 were positive and 24 negative in this test. One of these two positives was subsequently found to have VN antibody to BLU-1.

All animals exposed to BLU-20 and serially bled (group B) developed antibody which was detected in this test 12–20 days post-inoculation. Within bleeding intervals of 3–7 days there was no difference between the time of development of this GDP antibody and BLU-20 VN antibody.

Antigens and antisera of the other two Australian BLU viruses, serotypes 1 and 21 (group C), reacted in this test while antisera of the PAL group viruses (group D) and preparations from non-infected cells did not.

In the comparison of DR and SR forms of this test, 58 sera reacted in both, 12 in the DR only, 8 in the SR only, and 30 were negative in both. All 20 of the sera that reacted in one test only did so at the marginal '1' strength and all were from herds which contained animals which reacted in both tests.

GDP Test for BLU Based on the Deposit Antigen

When cell deposits were diffused against the five BLU-20 antisera, two potentially useful precipitin reactions, one of which could be identified with that of the group test, occurred consistently. A third zone of precipitation was too close to the antigen well to be useful as the basis for a GDP test. By electron microscopy, it was shown to be composed of virion or near-virion particles, whereas no morphologically identifiable material was demonstrable in the other two lines.

In the case of one bovine serum the two lines of interest were clearly separated and it was possible, by either of the methods described, to selectively remove the group-specific line and set up a test based on the second line only. The results obtained with this test (Table 1) indicate type specificity.

In group A, 25/26 of the sera which were positive in the VN test were also positive, while all VN negative sera, including the two group positive samples, were also negative, in this test.

In group B, of 59 sera derived from serial bleeds of animals experimentally exposed to BLU-20 virus, antibody was detected in 36, including the 34 samples that were positive in both VN and group GDP tests. The validity of the extra two type GDP reactions was indicated by positive reactions on subsequent sera from the same animals in the other two tests.

In group E, all of the 47 sera from the north coast of New South Wales, which were positive in the group BLU GDP test and negative by BLU-20 VN test, were also negative by type GDP test.

Additionally, antigens of, and sera against, the other BLU viruses (group C) and the PAL viruses (group D) did not react in this test.

GDP Tests for PAL Group Viruses

When supernatant antigen from M3 infected cells was diffused against convalescent serum from the sentinel cow, from which the virus was originally isolated, one precipitin line appeared. When formed by optimal concentrations of reactants, this provided the reference line for a GDP test. All antigens and antisera of the three PAL viruses reacted in this test. As in the case of BLU, this test is regarded as group-specific. However, the two calves, from which the initial BLU-20 reference serum was selected, also developed weak reactions in this test after the experimental BLU-20 infection, suggesting some cross reactivity.

When deposit antigen from M3 infected cells was diffused against the same serum, two precipitin lines formed. In a test based on double reference lines, antigen of both CSIRO Village and D'Aguilar viruses, and antisera to these and Bunyip Creek virus, all gave a turn of identity to one of these lines. This line was shown to be identical to that of the group test. Only Bunyip Creek antiserum had antibody identifiable with that of the second M3 line (Fig. 1).

All of the calves inoculated with PAL group viruses developed homologous VN antibody within 3 weeks of exposure. All developed strong PAL group GDP antibody that was boosted by their subsequent exposures to other PAL group viruses. From this series, 106/108 sera with VN antibody to one or more PAL group viruses contained antibody demonstrable in the PAL GDP group test. The two negative samples were from one animal within 4 weeks of its first exposure. None of the inoculated calves developed demonstrable BLU group antibody.
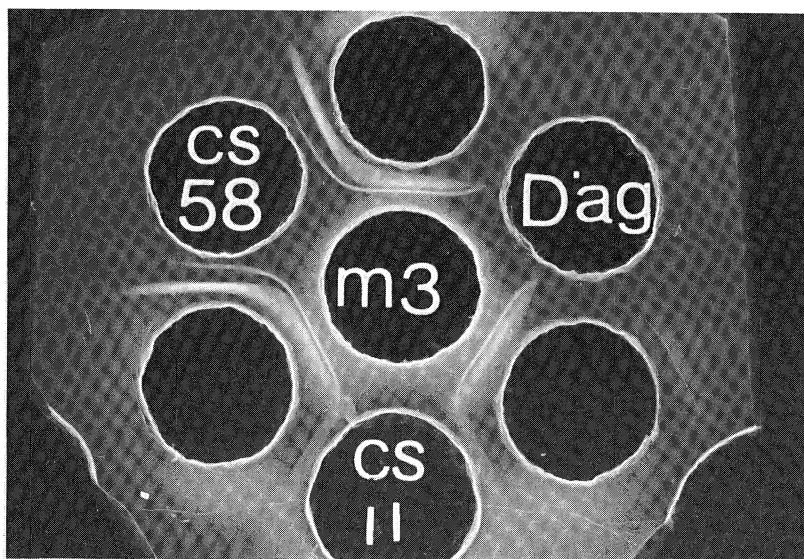

Fig. 1.   Double-line test based on Palyam group virus M3* antigen. Unlabelled wells, M3* cell deposit derived antigen; m3, bovine antiserum to M3* virus; D'ag, rabbit antiserum to D'Aguilar virus; CS11, rabbit antiserum to CSIRO Village virus; CS58, rabbit antiserum to Bunyip Creek virus. (*M3, Bunyip Creek serotype.)

Discussion

Two different GDP tests were developed using antigens derived from BLU-20 infected cell cultures. From the results presented one could be concluded to be BLU group-specific and the other BLU-20 type-specific.

The group-specific test was capable of detecting antigens of other Australian BLU viruses, BLU-1 and BLU-21, as well as that of BLU-20, and precipitating antibody in sera containing neutralizing antibody to any of these three viruses. Its sensitivity in detecting antibody to BLU-20, resulting from either natural or experimental infection (groups A and B), appeared to be comparable to that of the VN test. Within the scope of these observations specificity also appears to be high. Antigen preparations from viruses of the PAL group and sera of four experimental animals that had been serially exposed to three viruses of the PAL group failed to react in the test. In groups A and B there was only one serum that could be construed as possibly falsely reactive, although, with hindsight, exposure of the BLU-20 VN negative animals of group A to other BLU viruses, and, by analogy, probably to other

orbiviruses that may be potentially cross reactive, appears to have been remarkably limited. It should also be noted that in the wider field usage of the test in northern areas of New South Wales, reported in accompanying papers (Burton and Littlejohns 1988; Littlejohns and Burton 1988; Littlejohns *et al.* 1988), considerable circumstantial evidence suggested that many of the reactions observed, often weak and transient, were not attributable to BLU virus infections but were more likely to be the cumulative result of multiple exposures to related agents.

According to the comparison of DR and SR group tests, the results in the latter are equally valid with those in the former when used to test sera. There is no suggestion that the use of the field reference serum had compromised the specificity of the test for antibody and there is no theoretical reason to expect it to do so. However, the two tests could not be expected to be comparable when used to detect and identify antigen and only the DR form of the test should be used for that purpose.

The type-specific GDP test appears to have a sensitivity equal to or better than the BLU-20 VN test, and to be equally specific. It detected antibody in sera of calves and sheep 10–21 days after experimental infection, with reactivity in this test preceding the appearance of BLU-20 VN antibody on two occasions, by 3 and 13 days. Reactivity in both this and the VN test persisted for as long as observations were continued on these experimental animals (84 days). It also detected antibody in 25/26 VN reacting field sera. On the other
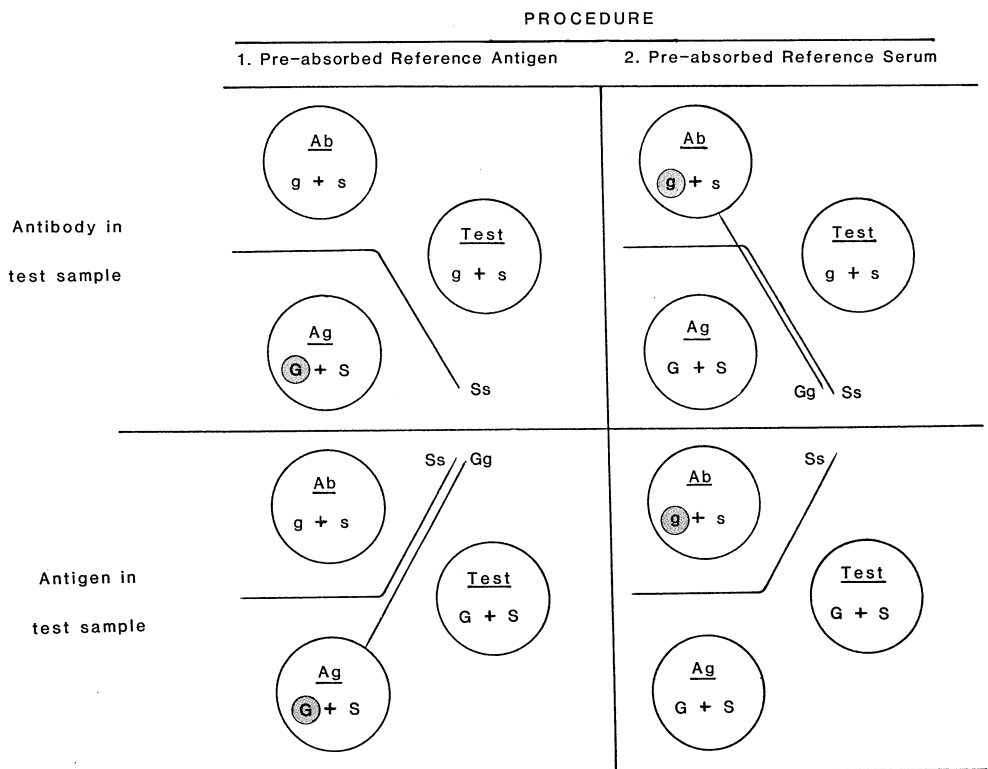
PROCEDURE

Fig. 2. Illustrating alternative procedures for preparing reactants to produce a single 'type' reference line, and consequent results in positive tests for antibody or antigen. Confusing 'group' precipitin lines between test material and a reference reactant are avoided if procedure 1 is used to generate a test for antibody and procedure 2 to generate a test for antigen. **Ab**, reference serum; **Ag**, reference antigen; **Test**, sample under test; **G**, group-specific antigen; **g**, antibody to G; (Shaded disc indicates activity removed by absorption.) **S**, type-specific antigen; **s**, antibody to S; **Gg, Ss**, precipitin lines, group- and type-specific respectively.

hand, no antibody was detected in sera of calves after experimental infection with other BLU viruses, nor in 47 sera from cattle in an area where BLU-20 VN antibody has never been detected, even though the latter samples were selected because they were reactive in the BLU group test. The type test did not detect antigen in cell deposit preparations from cell culture, infected by other BLU viruses, which reacted as antigen in the group test.

The basic reference materials from which the type-specific reaction is obtained, both serum and antigen, invariably also contains group-specific reactivity. Absorption of the antigen by a serum having group, but no type, antibody is preferred to generate a test to detect antibody while the alternative, of absorbing group antibody with cell culture fluid in which only group antigen is demonstrable, is preferred to generate a test to detect antigen. In each case, the preferred method avoids the occurrence of confusing group-specific precipitation between the test material and a reference reactant (Fig. 2).

Although up to 10 polypeptides have been found after the dissociation of immune precipitates from BLU infected cell cultures (Huismans 1979; Grubman *et al.* 1983) the precipitate demonstrated by immunodiffusion is usually described as a single identity. However, it cannot be assumed that it is always the same antigenic identity that is demonstrated, or that antigens which appear to be identical by immunodiffusion may not vary in their minor determinants. A reaction of partial identity between supernatant and deposit antigens could be simulated by using a serum that allowed the two lines from the deposit antigen to overlie each other. However, the apparent spur is then from the deposit antigen and this is not easily reconciled with the reaction of partial identity described by Jochim (1976) in which the 'soluble' antigen was more complex than that which was 'cell-associated'. On the basis of its chemical composition, a 'type-specific' precipitation was concluded by Gumm and Newman (1982) to be of a subvirion antigen. This seems to be unlikely to be analogous to the type line described here, which was lacking in morphologically recognizable character, or to the virion-like aggregates that were demonstrated electron microscopically but were only found very close to antigen wells. Their third (P5) band in 'some experiments' may be a more likely match for our type line.

The finding of precipitin reactions of broader and narrower specificities between antigen and antiserum of a PAL group virus would indicate that group-specific and type-specific GDP tests could be developed for viruses of that group also.

The terms 'group' and 'type' have been used to describe the specificity of the antigens. However, the terms cannot be expected to be absolute. The apparent specificity of an antigen will be largely determined by its dominant epitopes but, after serial infections by related viruses, antibody responses to minor, shared epitopes will be boosted by the repeated exposures. The consequent enhanced cross reactivity is likely to be more evident in nature than can be demonstrated experimentally.

While the group antigen described in this paper is common to the three Australian BLU viruses, it has not been identified as a particular virus protein or with antigen derived from cell cultures infected with BLU viruses from other continents. Assuming that it is based on the major nucleocapsid protein, P7 (Huismans and Erasmus 1981), then lack of total identity with the group antigen of African BLU types may be anticipated. The P7 protein is thought to be translated from dsRNA segment 7 (Huismans and Bremer 1981; Grubman *et al.* 1983). Although dsRNA segment 7 of BLU-20 hybridizes with that of African BLU-4, the homology is rather less than that expected between two different African serotypes (Huismans and Bremer 1981). Likely minor diversity in the nucleocapsid protein of BLU virus is also illustrated by that which has been demonstrated in VP9 (=P7) of American isolates by their having different patterns of monoclonal antibody binding (Appleton and Letchworth 1983). The serotype-common nature of the P7 antigens would appear to reflect the ease with which common, rather than unique, epitopes are demonstrated rather than reflecting a state of total homology.

The specificity of the GDP test, when used to detect antibody, is governed by the reference antigen. Hence, the precise epitopic spectrum of the major nucleocapsid protein

of the isolate used to produce that antigen will critically qualify both the interpretation of the results of tests for antibody and the nature of inter-group relationships inferred from such tests.

The type-specific antigen described also cannot be assumed to be absolute in that regard, any more than is the VN test with which the GDP test has been compared. Direct VN cross reactions do occur, for example between types 4, 17 and 20 (Erasmus et al., cited by Huismans and Bremer 1981) and this cross reactivity is seen in the immune-precipitation of P2 from various serotypes (Huismans and Bremer 1981). Also, serial infections can result in VN activity being generated against serotypes which have no demonstrable direct VN relationship to the infecting serotypes (Jeggo et al. 1983).

In practical terms, the empirical methods described here seem to provide for 'group-' and 'type-' specific GDP tests, subject to the exact definition of those terms. Purification of antigen and its separation from infective virus has been described and proposed as desirable to provide a safe antigen for a routine test (Gumm and Newman 1982; Hubschle and Yang 1983). The method described here for the group test may be equally satisfactory and present some advantage in simplicity. Purification of the antigen is not necessary because double diffusion in gel, alone among all serological techniques, can allow for the separate identification of a molecular antigenic identity within the test itself. Separation of antigen from infectious virus is also unnecessary because infectivity can be eliminated, without loss of antigenicity, by treatment with β-propiolactone. The practical usage of the type-specific test has not been as fully tested as has that of the group-specific test but it would seem to have some potential as an alternative to VN tests, with an expectation of similar qualifications in regard to cross reactions. In our experience, antigen yields, in terms of usable units, have been lower than those for the group test by a factor of about 10. As antigen production costs represent about 10% of the total cost of the group test, the total cost of the type test would be about twice that of the group test. This probably makes it competitive with VN and it has the added advantage that sterile samples and facilities for cell culture are not required.

Acknowledgment

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