Monoclonal antibody characterization of South African field isolates of *Haemophilus paragallinarum*

R. R. BRAGG[1], L. COETZEE[1] and J.A. VERSCHOOR[2]

ABSTRACT

BRAGG, R.R., COETZEE, L. & VERSCHOOR, J.A. 1993. Monoclonal antibody characterization of South African field isolates of *Haemophilus paragallinarum*. *Onderstepoort Journal of Veterinary Research,* 60: 181–187

A total of 27 different isolates of *Haemophilus paragallinarum* were made from chickens between June 1991 and December 1992. All of these isolates were examined by ELISA, by means of a locally produced panel of three monoclonal antibodies (denoted F1, V1 and VF3). The isolates were all of the F1 antigenic type. Three of them showed a weak reaction with the F1 monoclonal antibody, while three other isolates reacted strongly with the F1 as well as with the VF3 Mab.

A selection of stored *Haemophilus* isolates, dating from 1984 to 1985, were also examined with the Mabs and found to be of the F1 antigenic type. Fifteen isolates were collected before 1974, i.e. before the use of *Haemophilus* vaccines in this country. The majority of them were of the F1 antigenic grouping. Some showed a weak reaction with the F1 Mab; others showed a strong reaction with both the F1 and VF3 Mabs; and a few showed no significant reaction with any of the Mabs used.

Strains used for the production of infectious coryza vaccine were also examined with the Mabs. Strain 0083 showed a stronger reaction with the V1 Mab than with the F1 Mab, whereas strain 0222 showed no reaction with any of the Mabs.

None of the SA field isolates collected since the use of vaccines exhibits the V1 antigenicity, which is the prevalent antigen of strain 0083. Most (80 %) of the SA field isolates showed a stronger reaction with the F1 Mab than did strain 0083. Antigenically silent isolates similar to 0222 (Page's serotype B) were isolated before the use of vaccines, but not since.

INTRODUCTION

Haemophilus paragallinarum has been identified as the causative agent of infectious coryza of poultry (Yamamoto 1984), which has caused severe economic losses in the egg industry in many parts of the world. The disease is widespread in South Africa and continues to be a severe problem, despite the widespread use of infectious coryza vaccines. The bacterium was first isolated in the 1930s (De Blieck 1932) and was termed *H. gallinarum* (Elliot & Lewis 1934). The organism has subsequently been reclassified as *H. paragallinarum* as it has been found that isolates from chickens do not require X-factor for growth (Blackall & Reid 1982).

Substantial work has been done on the serological characterization of *H. paragallinarum*. The first work on the serological differentiation between different strains of *H. paragallinarum* was done by Page (1962), who used a plate agglutination method. He detected three different serotypes which were termed A, B and C. In an independent study in Japan (Kato & Tsubahara 1962), in which an agglutination

[1] Department of Poultry Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, 0110 South Africa

[2] Department of Biochemistry, University of Pretoria, Pretoria, 0001 South Africa

Received 14 June 1993–Editor

test was also used, three agglutinin serovars were recognized. The technique of Page has been widely used for the serological classification of *H. paragallinarum*.

Hinz (1980) developed a scheme based on heat-stable antigens detected in a gel diffusion test which enabled detection of six different antigenic serotypes. Hitherto, this method has not been widely used for the serotyping of *Haemophilus*.

Kume, Sawata, Nakase & Matsumoto (1983) established a scheme based on haemagglutination antigens obtained by potassium thiocyanate extraction and sonication. Gluteraldehyde-fixed chicken erythrocytes were used to carry out the haemagglutination test (HA). In this way, three different serogroups consisting of seven different serovars were identified. The serogroups were termed I, II or III and the serovars were termed HA-1 to HA-7. Serovars HA-1 to HA-3 were found to belong to serogroup I; serovars HA-4 to HA-6, to serogroup II; and serovar HA-7, to serogroup III. It was established that these serogroups were the same as Page's (1962) serogroups A, C and B, respectively (Sawata, Kume & Nakase 1980).

Blackall, Eaves & Rogers (1990b) proposed an alternative nomenclature to the Kume scheme, allowing for the addition of new serovars as they are discovered. The serovars, according to Blackall *et al.* (1990b), are termed A-1 to A-4, C-1 to C-4 and B-1. Blackall *et al.* (1990b) added two new serovars to the Kume scheme.

Page's serotype B strains were found to be untypable when tested by Kume, Sawata & Nakase (1980) and Sawata, *et al.* (1980). They were considered to be variants of serotype A or C strains that had lost their type-specific antigens. Serotype B isolates have, however, been found to be pathogenic and to constitute a distinct serotype (Jacobs, Cuenen & Strom 1992; Blackall, Eaves & Aus 1990).

Polyclonal antibodies were used to carry out all of the serological work mentioned above. Verschoor, Coetzee & Visser (1989) were the first to establish a panel of monoclonal antibodies against H. *paragallinarum* raised against strains 0083 (Page's serotype A) and 0222 (Page's serotype B) and two South African field isolates (isolate M 85 obtained in 1985 and isolate SB 86, in 1986) from diseased birds from flocks vaccinated with strain 0083 and 0222. The objective of establishing this panel of Mabs was to detect antigenic differences between "vaccine" strains and "field" isolates; they were not initially established to investigate the polyclonal serotyping work reviewed above.

Other monoclonal antibodies against *H. paragallinarum* have subsequently been established (Yamaguchi, Takigami, Iritani & Hayashi 1990a; 1990b). The usefulness of these monoclonal antibodies was evaluated (Blackall, Yamaguchi, Iritani & Rogers 1990c; Blackall, Zheng, Yamaguchi, Iritani & Rogers 1991). It was found that one of the Mabs produced by Yamaguchi *et al.* (1990a) was able to recognize all four of the Kume serovars belonging to serogroup A, although serovar A-3 and A-4 had significantly lower haemagglutination inhibition (HI) titres than serovar A-1 and A-2. Three other Mabs (Yamaguchi *et al.* 1990a; 1990b) were required to detect the four different serovars of serogroup C. There appears to be substantial antigenic variation between the different serovars of serogroup C, and a lesser degree of antigenic variation between the serovars of serogroup A. Serogroup B strains were not recognized by any of the four Mabs tested (Blackall *et al.* 1990c; 1991).

The Mabs established by Verschoor *et al.* (1989) were capable of detecting differences between the strains used for vaccination and the two isolates made in 1985 and 1986 from diseased birds from vaccinated flocks. Verschoor *et al.* (1989) did not extend their work to distinguish between field isolates other than M85 and SB86. This report focuses on the use of these monoclonal antibodies in order to evaluate various recent field isolates of *H. paragallinarum*; but also selected field isolates made before the wide-spread use of vaccines against infectious coryza in South Africa.

MATERIALS AND METHODS

Bacteria

Field isolates

A total of 27 different isolates of *H. paragallinarum* were isolated from diseased layers submitted to the laboratory between June 1991 and December 1992. Bacterial isolations were made from chickens showing symptoms of infectious coryza according to the techniques (without modifications) used by Bragg, Coetzee & Verschoor (1993). Samples were collected from sinuses and inoculated onto two blood tryptose agar (BTA) plates, one of which was streaked across the inoculum with a sample of *Staphylococcus aureus*. Both plates were incubated in a candle jar at 37 °C for 18 h. Any bacterial growth observed on the plate streaked only with the *S. aureus*, was tested for catalase, examined with the Mabs, and stored in the Microbank system (Davies Diagnostics, Randburg, South Africa).

A total of 15 freeze-dried isolates, made in the pre-vaccination-era (1972–1973), by Drs J.H. du Preez and S.B. Buys (at that time, both from the Veterinary Research Institute, Onderstepoort), were reconstituted and grown as described above. Two

recent field isolates (M85 and SB86, isolated from diseased birds from vaccinated flocks by L. Coetzee, Golden Lay Laboratories, 1985–1986) were kindly supplied by Golden Lay Laboratories. Isolate A505/84, made in 1984 by Dr F. Huchzermeyr (Veterinary Research Institute, Onderstepoort), was also reconstituted.

Vaccine strains

Strains 0083, 0222 and Modesto were obtained from our own stored sample collection, Golden Lay Laboratories (Verwoerdburg, South Africa) and from Dr P.J. Blackall (Animal Research Institute, Yeerongpilly, Australia). Stored samples, both in liquid nitrogen as well as freeze-dried samples in this laboratory, were reconstituted.

These isolates were inoculated into liquid modified Casman's medium (Coetzee, Rogers & Velthuysen 1983), at a pH of 7,4 and incubated at 37 °C. Samples were formalin-inactivated and examined by ELISA according to the methods used by Bragg *et al*. (1993).

Pasteurella *spp.*

A total of 12 different *Pasteurella* species, isolated from diseased chickens in this laboratory between June 1991 and December 1992, were selected. The isolates were identified by conventional biochemical procedures. These isolates were each made up of four isolates of *P. multocida*, *P. gallisepticum* and *P. haemolytica*.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The different isolates of *Pasteurella* spp. were inoculated into Casman's medium with serum and incubated at 37 °C for 24 h. The isolates were inactivated, washed and used to coat ELISA plates according to the methods used for the *Haemophilus* spp. The ELISA test was performed on the various *Haemophilus* isolates and *Pasteurella* spp., as described elsewhere (Bragg *et al*. 1993). Mabs F1, V1 and VF3, developed by Verschoor *et al*. (1989), were used. Culture fluid from the non-producing SP2 myelomas were used as background control.

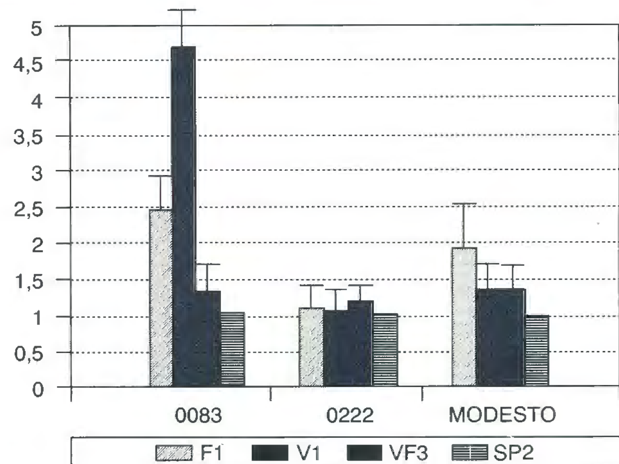
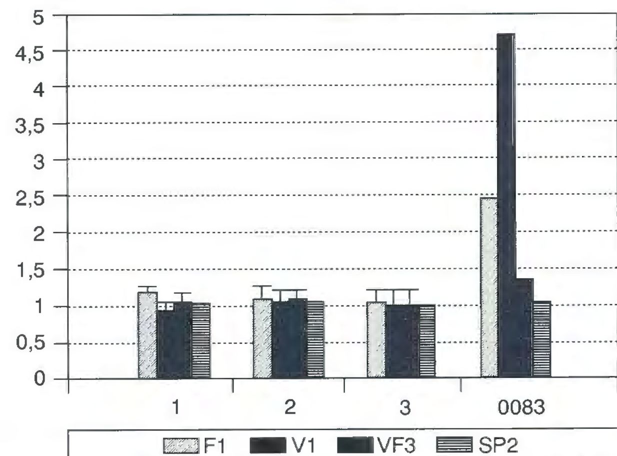


FIG. 1 Monoclonal antibody reaction patterns obtained with strain 0083, strain 0222 and Modesto. All readings are the signal to background ratio, which is calculated by dividing the OD signal of each Mab with the signal obtained for the background control (SP2). The standard deviation on each strain represents the difference between the three different sources of the bacteria



1 = *Pasteurella haemolytica*
2 = *P. gallinarum*
3 = *P. multocida*

FIG. 2 Mab reaction patterns obtained when 12 isolates of *Pasteurella* spp., made from the sinuses of diseased chickens, were examined with the panel of Mabs. Strain 0083 was used as a reference strain. All readings are the signal to background ratio, which was calculated in the same way as for Fig. 1

RESULTS

The monoclonal antibody patterns obtained when *H. paragallinarum* strains 0083, 0222 and Modesto, (taken from three different sources) were examined by ELISA, can be seen in Fig. 1. Strain 0083—from all three sources—showed a strong reaction with the V1 Mab and a weaker reaction with the F1 Mab. Strain 0222—from all three sources—showed virtually no reaction with any of the Mabs used. Modesto showed a weak reaction with the F1 Mab. No significant differences in the signal to background ratios for the different Mabs could be detected among the same strain from different sources.

No reaction with any of the Mabs was detected when the ELISA plates were coated with the three different *Pasteurella* spp. (Fig. 2).

From Fig. 3 it can be seen that the isolates of *H. paragallinarum*, made from diseased chickens between

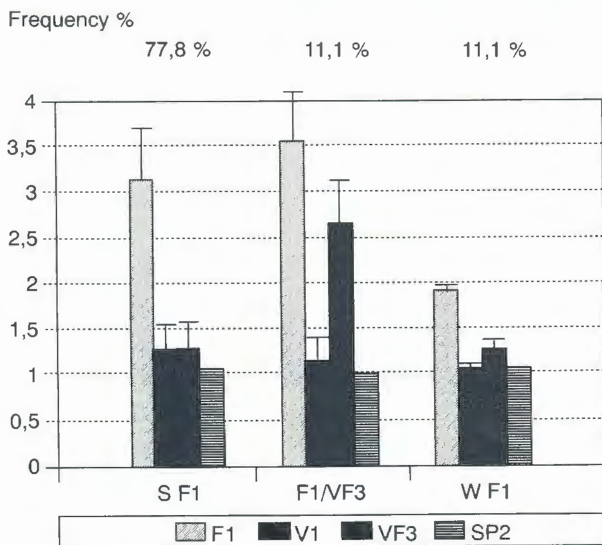


FIG. 3 Mab reaction patterns obtained when 28 different field isolates, made during 1991 and 1992 were examined with the panel of Mabs. Signal to background ratios were calculated in the same way as for Fig. 1

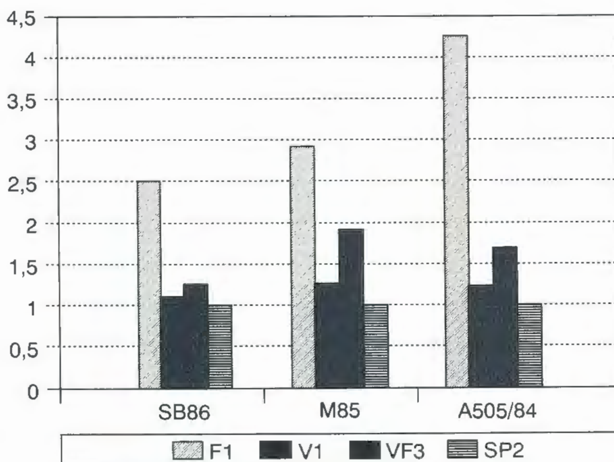
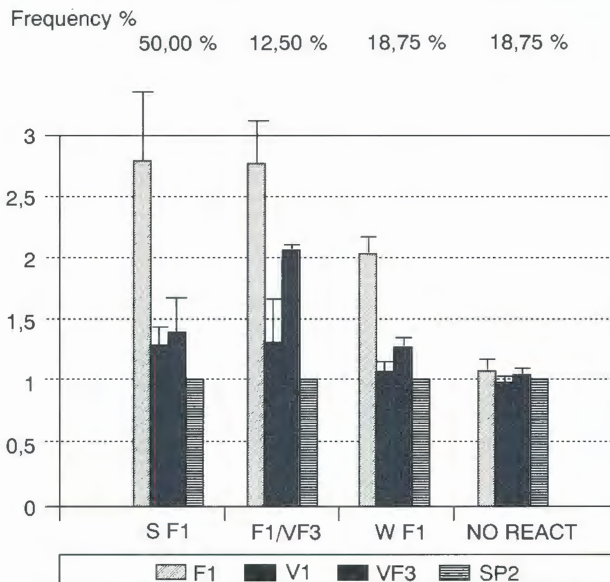


FIG. 4 Mab patterns obtained when three isolates, which were made in 1984–1986, were examined with the local panel of Mabs. The signal to background ratio was calculated in the same way as for Fig. 1

June 1991 and December 1992, fall into three distinct groups. The most prevalent group (designated SF1), consisting of 21 of the 27 isolates, showed a strong reaction with the F1 Mab and virtually no reaction with either the V1 or VF3 Mab. The second group (VF3/F1), consisting of three isolates, showed a strong reaction with the F1 as well as the VF3 Mab. The third group (WF1), also consisting of three isolates, reacted weakly with F1 and not at all with the other two Mabs.

According to the results in Fig. 4, the M85 isolate obtained in 1985 showed a reaction with the F1 as



S F1 = Strong reaction with the F1 Mab (> 2 x background)
F1/Vf3 = Strong reaction with the F1 and VF3 Mabs
W F1 = Weak reaction with the F1 Mab (< 2 x background)

FIG. 5 Mab reaction patterns obtained when 16 isolates of *H. paragallinarum*, which were made in 1972–1973 before vaccines against infectious coryza were used, were examined with a local panel of Mabs. The signal to background ratio was calculated in the same way as for Fig. 1.

well as the VF3 Mab, thereby appearing to be of the VF3/F1 group, while the isolates from 1984 and 1986 showed a strong reaction with the F1 Mab only, which is typical of the SF1 group.

Four groups could be established (Fig. 5) when the pre-vaccination-era isolates were examined. Three of these groups corresponded to the SF1, WF1 and VF3/F1 groups found with the new isolates (Fig. 3). The fourth group, found in the pre-vaccination isolates, corresponded to the Mab pattern found with strain 0222 (Fig. 1), i.e. there was no reaction with the three Mabs used.

DISCUSSION

The Mabs F1, V1 and VF3 appear to be quite specific for *H. paragallinarum*, as no significant signal could be observed when three species of the closely related genus, *Pasteurella*, were used as antigen.

The reaction patterns obtained when reference strains 0083, 0222 and Modesto were tested, were significantly different from each other (Fig. 1). Strain 0222 did not react with any of the three Mabs tested in these experiments. This work corresponds to the findings of Yamaguchi, *et al.* (1990a) and Blackall *et al.* (1990c; 1991). They found that none of the Page's serotype B organisms reacted with their monoclonal antibodies. These results, however,

differ from those of Verschoor *et al.* (1989) who found a strong reaction with the V1 Mab when testing strain 0222. This reaction with the V1 Mab was found in only one batch of strain 0222, which was grown in bulk, inactivated, stored, and never again found on subsequent batches. The V1 Mab was found to react strongly only with strain 0083. In these experiments, strain 0083 also showed a significant reaction with the F1 Mab (Fig. 1). This again differs from Verschoor *et al.* (1989) who found no reaction with the F1 Mab on 0083. The Modesto strain showed no reaction with either the V1 or VF3 Mab; but showed a weak reaction with the F1 Mab.

From these results it would appear that there are three distinct antigenic types that can be detected with the South African panel of Mabs. These antigenic types appear to correspond to Page's A (strain 0083), which is referred to as the V1 antigenic type; Page's B (strain 0222), which is referred to as a non-reactive antigenic type; and Page's C (Modesto), which is referred to as the F1 antigenic type.

Three different antigenic groupings can be distinguished (Fig. 4) when the results of the examination of recently isolated field isolates of *H. paragallinarum* with the Mabs are examined. The F1 antigen is expressed in all three of these groupings: one group, consisting of 11 % of the isolates, has a weak reaction with the F1 Mab, while another group, consisting of 78 % of the recently isolated field isolates, had a strong reaction with the F1 Mab. A third group, consisting of 11 % of the isolates, reacted strongly with the F1 Mab as well as with the VF3 Mab. This group is of particular interest in cancer research, in that the antigen recognized by the VF3 Mab appears to be associated with the induction of spontaneous fusion between *Haemophilus* immune murine B-cells and myeloma cells (Verschoor, Boshoff, Coetzee, Van Wyngaardt & Visser 1990). Boshoff, Coetzee, Visser & Verschoor (1992) established that the VF3 Mab recognizes a protease-resistant antigen which can be extracted by a hot phenol procedure used typically for lipopolysaccharide extraction. The F1 Mab recognizes a protease-sensitive antigen (Boshoff 1991).

All three of the stored isolates, made in 1984–1986, reacted strongly with the F1 Mab. Two of these isolates also showed a strong reaction with the VF3 Mab. Although there are insufficient samples of these isolates to make conclusive observations, it should be noted that both of the antigen groupings found in these three old isolates were also found in the new isolates of *H. paragallinarum*, i.e. SF1 and the VF3/F1 antigenic groupings.

Interesting results were obtained when the isolates made before the use of vaccines in South Africa were examined with the Mabs. Of the 16 isolates examined, 13 showed a reaction with the F1 Mab. Of these, 50 % showed a strong reaction with F1 and no reaction with the V1 or VF3 Mab. This dominant group corresponded directly to the majority of the recent isolates designated SF1. Similarly, 12 % of the pre-vaccination isolates showed a reaction with both the F1 and the VF3 Mabs, correlating with the 11 % of the recent isolates which showed the VF3/F1 pattern. A total of 18 % of the pre-vaccination isolates showed the WF1 pattern which was observed with 11 % of the recent isolates. Three antigenic groups in the pre-vaccination isolates therefore correspond well to the three groups of the recent isolates. There is, however, a fourth antigenic group amongst the pre-vaccination isolates, comprising 18 % of these isolates. It was found that this group did not react with any of the Mabs tested. This corresponds to the Mab pattern of strain 0222 (Fig. 1). It is interesting to note that none of the recent field isolates from vaccinated flocks had a Mab pattern similar to that of 0222.

None of the 27 field isolates from 1991–1992, the three older field isolates (1984–1986) or the 16 pre-vaccination era (1972–1973) isolates showed any reaction with the V1 Mab. It can therefore be concluded that none of the 46 field isolates of *H. paragallinarum* isolated from chickens in South Africa possessed the V1 antigen. This antigen is the most abundant antigen produced by strain 0083 and can be detected with the panel of Mabs used in these experiments. Strain 0083 also expresses the F1 antigen, which predominates in South African field isolates.

The high levels of expression of the F1 antigen in all the post-vaccination-era isolates in South Africa clearly differ from the lower level of expression in strain 0083 and the non-expression in strain 0222. As has been seen in South Africa, infectious coryza still occurs in vaccinated chickens but, significantly, these cases do not constitute complete failure of the vaccine—many of the vaccinated flocks remained at least partially protected against infectious coryza. It is interesting to note that 18 % of the isolates made before the use of vaccines exhibited a Mab pattern similar to that of 0222. None of the isolates made after the use of vaccines have such a Mab pattern. It can therefore be postulated that *H. paragallinarum* —which is antigenically similar to strain 0222—occurred in South Africa, and that this serotype has been effectively selected against by the use of vaccines.

Although the F1 antigen is the prevailing antigenic type of *H. paragallinarum* in South Africa, it should be noted that strain 0083 also expresses the F1 antigen at significant levels. If the F1 antigen plays any role in the immunogenicity of the organisms, chickens vaccinated with strain 0083 should have some measure of protection against organisms expressing the F1 antigen. It must be noted that 80 %

of the South African field isolates of *H. paragallinarum* exhibit a stronger reaction with the F1 Mab than does strain 0083. It is possible that the level of immunity stimulated by the F1 protein of strain 0083 is insufficient to fully protect against strains which produce higher levels of the F1 protein. This aspect needs further investigation.

It has been well established that the L antigen (Kume *et al.* 1980) and the HA-L antigen (Sawata, Kume & Nakase 1982), isolated from serotypes I and II of *H. paragallinarum*, each plays a role in protective immunity. The L antigen was found to be heat labile and trypsin sensitive, thus indicating that this antigen is a protein. The relationship between the L antigen of Kume *et al.* (1980) and the antigen reacting with the F1 Mab, is currently under investigation and will be reported on in due course.

Recently, NAD-independent *H. paragallinarum* organisms were isolated from chickens in South Africa (Bragg *et al.* 1993). Groupings that are similar to those found with the normal field isolates, were found in the NAD-independent isolates. There were only two significant differences: none of the NAD-independent isolates showed any significant reaction with the VF3 Mab, while four isolates from layers and three from broilers showed a reaction with the V1 Mab. As already indicated, none of the typical field isolates showed any reaction with the V1 Mab. At this stage it is not yet clear why a few of the NAD-independent isolates from chickens in South Africa were found to produce the V1 antigen (the predominant antigen of strain 0083), while none of the typical isolates examined before and after the use of vaccines in this country was found to do so. Possibly the presence or absence of a plasmid (or plasmids) may have some role to play in the expression or suppression of various antigens in *Haemophilus paragallinarum*.

When the plasmid coding for NAD independence was inserted into strain 0083, the expression of the V1 antigen was inhibited—as seen by the lack of the V1 antigen in the NAD-independent, transformed strain 0083 (Bragg *et al.* 1993). The role of plasmids in the expression of antigens in *H. paragallinarum* requires further investigation.

The biochemical nature and function of the V1 antigen and of the other antigens detected by our Mabs in immunogenicity, as well as their virulence, are at present under investigation.

ACKNOWLEDGEMENTS

The authors are grateful to the South African Cancer Association and the Egg Board for financial contributions; to Dr P.J. Blackall and Golden Lay Laboratories for kindly supplying them with *H. paragallinarum* isolates; and to S. van Wyngaard, J. Carstens and A.J. Davis for expert technical assistance.

REFERENCES

BLACKALL P.J., EAVES, L.E. & AUS G. 1990a. Serotyping of *Haemophilus paragallinarum* by the Page scheme: comparison of the use of agglutination and hemagglutination-inhibition tests. *Avian Diseases*, 34:643–645.

BLACKALL, P.J., EAVES, L.E. & ROGERS, D.G. 1990b. Proposal of a new serovar and altered nomenclature for *Haemophilus paragallinarum* in the Kume hemagglutinin scheme. *Journal of Clinical Microbiology*, 28:1185–1187.

BLACKALL, P.J., YAMAGUCHI, T., IRITANI, Y. & ROGERS, D.G. 1990c. Evaluation of two monoclonal antibodies for serotyping *Haemophilus paragallinarum*. *Avian Diseases*, 34:861–864.

BLACKALL, P.J., ZHENG, Y-Z., YAMAGUCHI, T., IRITANI, Y. & ROGERS, D. G. 1991. Evaluation of a panel of monoclonal antibodies in the subtyping of *Haemophilus paragallinarum*. *Avian Diseases*, 35:955–959.

BLACKALL, P.J. & REID, G.G. 1982. Further characterization of *Haemophilus paragallinarum* and *Haemophilus avium*. *Veterinary Microbiology*, 7:359–367.

BOSHOFF, C.H. 1991. Spontaneous cell fusion induced by bacterial immunization. M.Sc. Thesis, Faculty of Science, University of Pretoria. South Africa.

BOSHOFF, C.H., COETZEE, L., VISSER, L. & VERSCHOOR, J.A. 1992. Spontaneous hybridoma formation induced by immunization with *Haemophilus paragallinarum*: evidence for a lipopolysaccharide fusion inducer. *Hybridoma*, 11:257–266.

BRAGG, R.R., COETZEE, L. & VERSCHOOR, J.A. 1993. Isolation of NAD independent *Haemophilus paragallinarum* from commercial chickens in South Africa. *Onderstepoort Journal of Veterinary Research*, 60:147–152

COETZEE, L., ROGERS, E.J. & VELTHUYSEN, L. 1983. The production and evaluation of a *Haemophilus paragallinarum* (infectious coryza) oil emulsion vaccine in laying birds. *Diseases prevention and control in poultry production. International Union of Immunological Societies Proceedings,* 66: 277–283.

DE BLIECK, L. 1932. A haemoglobinophilic bacterium as the cause of contagious catarrh of the fowl. *Veterinary Journal*, 88:9–13.

ELLIOT, C. & LEWIS, M.R. 1934. A hemophilic bacterium as a cause of infectious coryza in the fowl. *Journal of the American Veterinary Medical Association*, 37:878–888.

HINZ, K-H. 1980. Heat-stable antigenic determinants of *Haemophilus paragallinarum*. *Zentralblatt für Veterinaermedizin. Reihe B*, 27:668–676.

JACOBS, A.A.C., CUENEN, W. & STROM, P.K. 1992. Efficacy of a trivalent *Haemophilus paragallinarum* vaccine compared to bivalent vaccines. *Veterinary Microbiology*, 32:43–49.

KATO, K. & TSUBAHARA, H. 1962. Infectious coryza of chickens. II. Identification of isolates. *Bulletin of the National Institute for Animal Health*, 45:21–26.

KUME, K., SAWATA, A. & NAKASE, Y. 1980. Immunological relationship between Page's and Sawata's serotype strains of *Haemophilus paragallinarum*. *American Journal of Veterinary Research*, 41:757–760.spn

KUME, K., SAWATA, A., NAKASE, Y. & MATSUMOTO, M. 1983. Serological classification of *Haemophilus paragallinarum* with a hemagglutinin system. *Journal of Clinical Microbiology,* 17: 958–964.

PAGE, L.A. 1962. *Haemophilus* infection in chickens. 1. Characteristics of 12 *Haemophilus* isolates recovered from diseased chickens. *American Journal of Veterinary Research*, 23: 85–95.

RIMLER, R.B., DAVIS, R.B. & PAGE, R.K. 1977. Infectious coryza: cross protection studies, using seven strains of *Haemophilus paragallinarum*. *American Journal of Veterinary Research*, 38:1587–1589.

SAWATA, A., KUME, K. & NAKASE, Y. 1980. Biological and serologic relationships between Page's and Sawata's serotyping of *Haemophilus paragallinarum*. *American Journal of Veterinary Research*, 41:1901–1904.

SAWATA, A., KUME, K. & NAKASE, Y. 1982. Hemagglutinin of *Haemophilus paragallinarum* serotype 2 organisms: Occurrence and immunologic properties of hemagglutinin. *American Journal of Veterinary Research*, 43:1311–1314.

VERSCHOOR, J.A., COETZEE, L. & VISSER, L. 1989. Monoclonal antibody characterization of two field strains of *Haemophilus paragallinarum* isolated from vaccinated layer hens. *Avian Diseases*, 33:219–225.

VERSCHOOR, J.A., BOSHOFF, C.H., COETZEE, L., VAN WYNGAARDT, S. & VISSER, L. 1990. Spontaneous fusion between splenocytes and myeloma cells induced by bacterial immunization. *Hybridoma*, 9:511–518.

YAMAGUCHI, T., TAKIGAMI, S., IRITANI, Y & HAYASHI, Y. 1990a. Characterization and use of monoclonal antibodies to identify *Haemophilus paragallinarum* serovars. *Avian Diseases*, 34:52–57.

YAMAGUCHI, T., TAKIGAMI, S., IRITANI, Y & HAYASHI, Y. 1990b. Characterization of two new monoclonal antibodies against *Haemophilus paragallinarum* serovar C hemagglutinating antigen. *Avian Diseases*, 34:922–927.

YAMAMOTO, R. 1984. Infectious coryza, in *Diseases of Poultry* (8th Ed.), edited by M.S. Hofstad, H.J. Barnes, B.W. Calnek, W.M. Reid, & E.R. Yoder Jr. Iowa State University Press: 178–186.