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Protection against experimental infection with influenza virus A/equine/Miami/63 (H3N8) provided by inactivated whole virus vaccines containing homologous virus

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SUMMARY

Thirty-one ponies immunized with inactivated virus vaccine containing A/equine/Miami/63 (H3N8) virus and six seronegative ponies were experimentally challenged with the homologous virus strain. All 6 unvaccinated ponies and 11 out of 31 vaccinated ponies became infected. A clear relationship between pre-challenge antibody, measured by single radial haemolysis (SRH), and protection was demonstrated as judged by virus excretion, febrile responses and antibody responses. Those ponies with SRH antibody levels > 74 mm^2 were completely protected against challenge infection by the intranasal route.

INTRODUCTION

Inactivated whole virus vaccines are widely used in the control of equine influenza. In previous studies (Wood *et al.* 1983*a*; Mumford *et al.* 1983) it was shown that the protection afforded by conventional, non-adjuvanted vaccines was short-lived, and that protection against intranasal challenge correlated with circulating antibody levels as measured by the single radial haemolysis (SRH) technique. In an experiment in which ponies, vaccinated with graded doses of the prototype H3N8 virus (A/equine/Miami/63) were challenged with a recent isolate, A/equine/Newmarket/79 (H3N8) only 2 out of 40 ponies (each with SRH antibody levels > 65 mm^2) were protected. From this original study it was not possible to establish whether the poor performance of the vaccine related to inadequate vaccine potency in terms of antigenic content or to antigenic differences between the vaccine and challenge strains of the H3N8 viruses used.

This paper describes a second vaccination and challenge experiment in which 31 ponies were vaccinated with graded doses of vaccine containing A/equine/Miami/63 and were subsequently challenged with the same H3N8 strain of virus in order to determine the level of antibody required for protection against challenge with homologous virus. The experiment was designed to

examine the relationship between vaccine potency and protection in the absence of antigenic differences between vaccine and challenge viruses.

The relationship between pre-challenge antibody to A/equine/Miami/63 virus as measured by SRH and protection was assessed by measurement of antibody responses, pyrexia and virus excretion following intranasal challenge with A/equine/Miami/63 virus.

MATERIALS AND METHODS

Challenge virus

The prototype H3N8 virus, A/equine/Miami/63, was obtained from the WHO World Influenza Centre, London, at the sixth passage level. It was further passaged in eggs (×5) to provide a challenge virus (titre 10^8 EID_{50}/ml; HA titre 32), which was used to infect two seronegative ponies. Although virus replication occurred and serological responses were demonstrated no clinical signs of infection (pyrexia or coughing) developed. However, when virus recovered from one of these ponies was transferred to the nasopharynx of a third seronegative pony, infection, with typical signs of influenza (pyrexia and coughing), was established. Virus recovered from nasopharyngeal swabs taken on the fourth day post-infection from the third pony was passaged once in eggs to provide a challenge virus for this experiment (10^8EID_{50}/ml) which was capable of inducing clinical signs of influenza.

Vaccines

Beta-propiolactone-inactivated A/equine/Miami/63 (H3N8) whole virus aqueous vaccines (provided by Duphar, Weesp, Holland) were standardized by single radial diffusion (SRD) (Wood *et al.* 1983*b*) to contain 1 μg, 5 μg, 15 μg or 50 μg haemagglutinin (HA) per dose.

Study design

Vaccination. The study was carried out in Welsh mountain pony yearlings which were seronegative for A/equine/Miami/63 as measured by the SRH test. Ponies were divided into four groups of eight ponies. Each group received vaccines of different antigenic content (1 μg, 5 μg, 15 μg and 50 μg). Each group of eight ponies was further subdivided into two groups of four ponies. One subgroup received two doses of vaccine with a 4-week interval between doses, the other subgroup received three doses of vaccine with intervals of 4 and 10 weeks between consecutive doses in order to generate a wide range of antibody levels at the time of challenge. Sera were taken at 2- to 4-week intervals during the course of vaccination. One pony in the group receiving two doses of vaccine containing 5 μg HA/dose was eliminated from the challenge study as it was difficult to handle.

Challenge. All vaccinated ponies, together with six unvaccinated control ponies, were challenged by intranasal instillation of 5 ml egg allantoic fluid containing $10^{8\cdot7}EID_{50}$ 13·5 weeks after the second dose and 3·5 weeks after the third dose of vaccine. This virus dose had been used successfully to reproduce clinical influenza in a previous study (Mumford *et al.* 1983).

Serum samples were collected prior to challenge and 28 days later. Rectal temperatures were recorded on the day of challenge and for the following 6 days between 15.00 and 17.00 h in order to minimize differences due to diurnal variations. Naso-pharyngeal swabs were taken daily for 6 days following challenge.

Serological test. The SRH test has been described previously (Wood *et al.* 1983*a*). Briefly, inactivated (56 °C; 30 min) sera (10 μl) were introduced into wells (3 mm diameter) cut in agarose (Seakem M.E.M., Miles Laboratories) containing sheep erythrocytes sensitized with virus in the presence of chromium chloride and fresh guinea-pig complement. Diffusion of sera in the plates was allowed for 20 h in a humidified chamber at 34 °C. Haemolysis zone diameters were measured in two directions at right angles using an Autodata® zone reader (Autodata, Hitchin, England).

Haemolysis zone areas were calculated and a significant increase in antibody was taken as a 50% increase in zone area. Control antisera were included on each plate and the test rejected if there was more than a $\pm 10\%$ difference in control zone areas on different test days.

Virus isolation. Nasopharyngeal swabs were maintained in virus transport medium on ice during transport from the isolation facilities to the laboratory. All swabs were frozen at −70 °C prior to virus isolation attempts. Exudate from each swab was diluted by 10-fold dilutions, and 0·1 ml from each dilution inoculated into the allantoic cavity of 10-day-old embryonated eggs (three eggs per dilution). The virus titre (EID_{50}/0·1 ml) in swab extracts was calculated from HA activity in allantoic fluids after 48 h incubation at 35 °C.

Analysis of results

Serology. Mean antibody responses were calculated for the groups of ponies receiving graded doses of vaccine.

Responses to challenge infection. Responses following challenge were analysed in relation to SRH antibody at the time of challenge. Vaccinated ponies were divided into four groups, with SRH antibody < 4 mm^2, 4–50 mm^2, 51–100 mm^2 or > 100 mm^2. The mean temperature response for each group was calculated as described previously (Mumford *et al.* 1983). A significant temperature was taken as $\geqslant 38{\cdot}9$ °C. Mean titres of virus shed were calculated as EID_{50}/0·1 ml swab extract/pony/day.

RESULTS

SRH antibody responses to vaccination

Fig. 1 illustrates the antibody responses following the first (V1), second (V2) and third (V3) doses of vaccines containing 1, 5, 15 and 50 μg HA/dose. After the second dose of vaccine, peak antibody responses were dose-related. Antibody levels declined rapidly after the initial boost, such that 6 weeks after the second dose of vaccine, antibody levels in those ponies receiving 1–5 μg HA/dose were barely detectable. Following the third dose of vaccine, given to four out of eight ponies in each group, antibody responses were generally higher than those stimulated by the second dose of vaccine, but were not dose-related (Fig. 1). At the time of challenge ponies which received two doses of vaccine had antibody levels

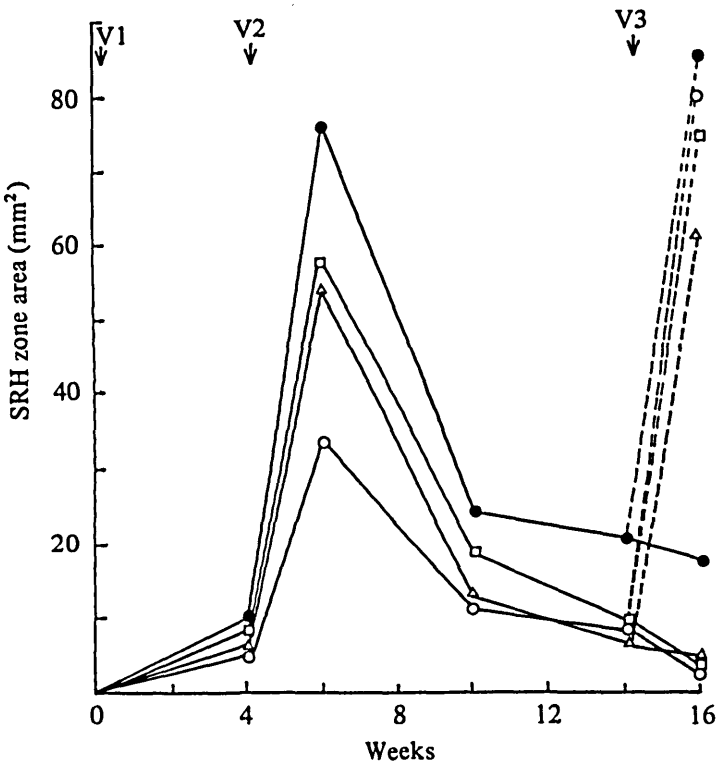

Fig. 1. Mean SRH antibody responses to one, two (—) or three (— — —) doses of vaccines containing 1 μg HA/dose (○), 5 μg HA/dose (△), 15 μg HA/dose (□) and 50 μg HA/dose (●).

of between 0 and 71 mm² zone areas whereas ponies which received three doses of vaccine had SRH zone areas 6–140 mm².

Responses to challenge infection

Responses, including pyrexia, antibody production and virus excretion, following challenge were analysed in relation to SRH antibody levels at the time of challenge (Table 1).

Febrile responses

Three out of six control ponies developed rectal temperatures ⩾ 38·9 °C 48 h after challenge. Three further control ponies showed increases in temperature of > 1 °C on the second day post-challenge, but peak temperatures recorded were < 38·9 °C. In the vaccinated groups of ponies the proportion of ponies developing significant temperatures (⩾ 38·9 °C) was related to the antibody levels at the time of challenge. Eighty per cent of ponies with SRH antibody < 4 mm, 57% of those with SRH antibody 4–50 mm², and 36% of those with antibody levels 50–100 mm² developed significant temperatures following challenge, whereas none of the ponies with SRH antibody levels > 100 mm² did so. There was no direct relationship between antibody and duration or height of febrile responses.

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Table 1. *Antibody responses, febrile reactions and virus excretion in ponies after challenge with A/equine/Miami/63 virus*

			Significant temperature responses†			Virus excretion		
Pony Group	Pre-challenge SRH antibody (mm^2) to Miami/63	Significant* antibody (%) rises	No. of ponies: No. in group (%)	Mean duration (days)	Mean significant temperature (°C)	No. of ponies: No. in group (%)	Mean duration (days)	Mean titre‡
Unvaccinated controls	< 4·0	6/6 (100)	3/6 (50)	2·3	39·27	6/6 (100)	> 6·0	$9·8 \times 10^4$
Vaccinates	< 4·0	10/10 (100)	8/10 (80)	1·4	39·44	10/10 (100)	4·4§	$4·8 \times 10^4$
	4–50	7/7 (100)	4/7 (57)	2·0	39·44	7/7 (100)	4·1§	$1·9 \times 10^4$
	51–100	3/11 (27)	4/11 (36)	2·1	39·16	10/11 (90)	2·6§	$4·1 \times 10^3$
	> 100	0/3 (0)	0/3 (0)	—	—	1/3 (33)	1§	$3·2 \times 10^0$

* 50% increase in area of haemolysis zone.
† Rise in temperature above 38·8 °C.
‡ EID_{50}/0·1 ml swab extract/pony/day.
§ Significant reduction in duration of virus shedding, $P < 0·001$.

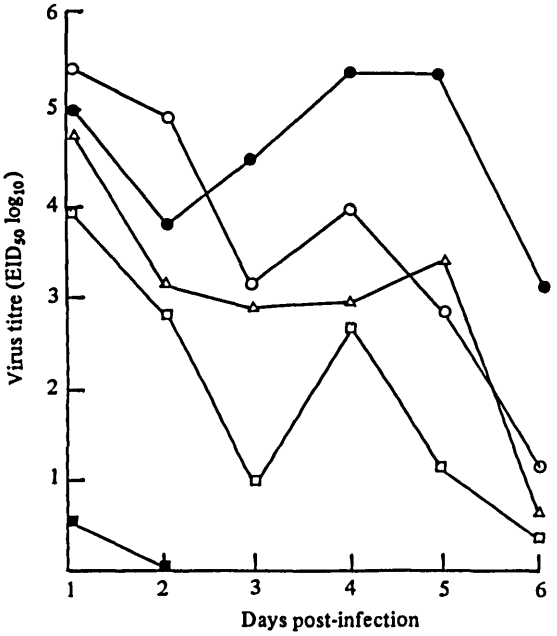

Fig. 2. Mean titres (EID_{50}/0·1 ml) of infectious virus (A/equine/Miami/63) in nasal swab extracts collected from vaccinated ponies with pre-challenge SRH antibody < 4 mm² (○), 4–50 mm² (△), 51–100 mm² (□), > 100 mm² (■) and unvaccinated ponies (●).

Virus shedding

All six unvaccinated control ponies shed virus, similarly virus was recovered from 10 out of 10 seronegative vaccinees. Of the 21 seropositive vaccinees 18 shed virus. Those ponies from which virus was not recovered had SRH antibody levels > 90 mm².

Virus excretion in unvaccinated controls continued for at least 6 days post-challenge. In all vaccinated groups, duration of virus excretion was reduced as compared with the controls ($P < 0{\cdot}001$). There was an inverse relationship between duration of virus shedding and level of pre-challenge antibody; for example ponies with antibody levels < 4 mm² shed virus for a mean of 4·4 days, whereas ponies with antibody levels > 100 mm² shed virus for no more than 1 day (Table 1).

The amount of virus shed by vaccinated ponies was less than that shed by unvaccinated controls. Within the vaccinated groups there was a clear inverse relationship between pre-challenge antibody levels and amount of virus shed (Table 1). Relatively large quantities of virus were recovered from vaccinated ponies with antibody levels of < 100 mm². This probably reflected, in part, recovery of challenge inoculum rather than replicating virus, since maximum titres of virus were recovered on the first day post-challenge (Fig. 2) and ponies were inoculated and sampled at the same site (nasopharynx). Replication and production of new virus peaked between 4 and 5 days post-challenge (Fig. 2).

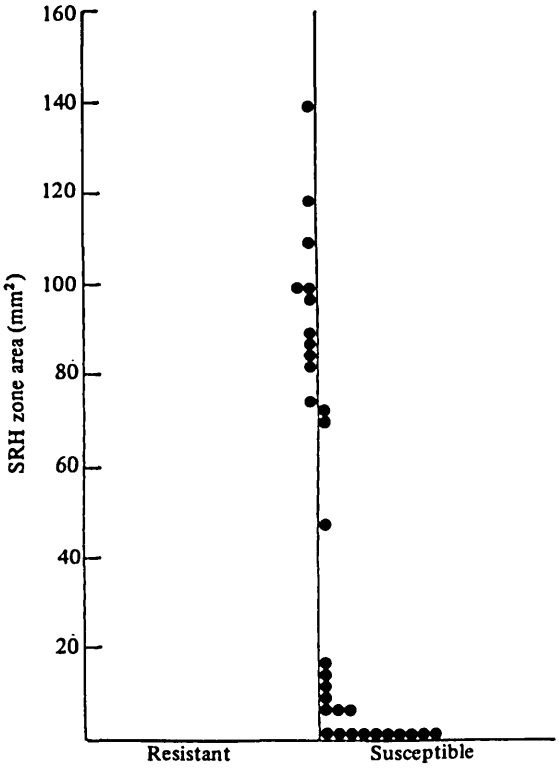

Fig. 3. Pre-challenge SRH antibody levels (mm²) in ponies which were either susceptible or resistant, as indicated by presence or absence of seroconversion following challenge infection with A/equine/Miami/63 (H3N8) virus given by the intranasal route.

Antibody responses

All six control ponies developed significant antibody responses to A/equine/Miami/63 (H3N8) following challenge, as did the 10 seronegative vaccinees. Of the seropositive vaccinees 7/7 with antibody levels < 50 mm² developed significant rises, whereas only 3/11 of those with antibody levels 51–100 mm² and 0/3 ponies with antibody levels > 100 mm² did so (Table 1).

All 15 ponies which had received 2 doses of vaccine prior to challenge seroconverted, whereas only 5 out of 16 ponies which received three doses of vaccine did so. None of the ponies with pre-challenge SRH antibody levels of > 74 mm² seroconverted following challenge (Fig. 3), whereas all those with antibody levels < 74 mm² did so.

Clinical signs of respiratory disease

Occasional coughing was observed in all the control ponies and some of the vaccinees, but no attempt was made to quantify this response. No febrile response or coughing was recorded in ponies with pre-challenge SRH antibody levels > 74 mm² except one pony which developed a temperature of 38·9 °C on day 6 post-infection, which was not attributed to the influenza challenge.

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DISCUSSION

This study was carried out to examine the relationship between vaccine-induced antibody (detectable by SRH) to A/equine/Miami/63 and protection provided against infection with homologous virus. The challenge system used (intranasal instillation of 5 ml of egg allantoic fluid containing $10^{8.7}EID_{50}$) was similar to that used successfully in a previous experiment in which ponies vaccinated with inactivated whole virus (A/equine/Miami/63) were challenged with a recent isolate of the H3N8 viruses, A/equine/Newmarket/79 (Mumford *et al.* 1983).

In the present study it was possible to calculate that an SRH zone size of 74 mm^2 was consistent with complete protection following challenge with this homologous virus, as demonstrated by the absence of clinical signs or seroconversion. The sensitivities of the SRH tests used in this and the previous study were similar (data not shown) and the implication is that the level of antibody required for protection against intranasal challenge with homologous virus is similar to that required for protection against challenge with a heterologous strain of H3N8 virus (62 mm^2) (Mumford *et al.* 1983). One possible explanation for this finding may lie with the challenge system used. In order to produce clinical symptoms in ponies challenged with A/equine/Miami/63 virus, it was necessary, first, to adapt the virus to ponies by serial passage and secondly, to infect intranasally with high levels of virus ($10^{8.7}EID_{50}$). Similar high levels of A/equine/Newmarket/79 virus were also used in the previous study. It is possible that these high dose levels obscured differences in the abilities of homologous and heterologous virus vaccines to protect against infection. Thus, in this experiment vaccine efficacy was a function of potency, and antigenic homology with challenge virus did not materially change the efficacy of the vaccine. Studies are in progress to develop alternative, less severe challenge procedures, which could be used to examine the efficacy of homologous and heterologous virus vaccines in parallel.

In the present study, the serological data demonstrated a clear relationship between vaccine potency (μg HA/dose) and levels of antibody stimulated by one and two doses of vaccine, confirming previous observations (Wood *et al.* 1983*a*) that the SRD technique (Wood *et al.* 1983*b*) was a useful *in vitro* technique for the measurement of vaccine potency. The protective level of antibody, calculated to be 74 mm^2, was stimulated after a second dose of vaccine in 6 of 8 ponies receiving 50 μg HA/dose, 4 of 8 receiving 15 μg HA/dose, 2 of 8 receiving 5 μg HA/dose and 1 of 8 receiving 1 μg HA/dose.

As in the previous study in ponies, the duration of antibody responses following the second dose of vaccine was short-lived, such that antibody was barely detectable 10 weeks after the second dose of vaccine in all except those ponies which received the highest dose (50 μg HA) of vaccine. The duration of protective levels of antibody was in all cases less than 4 weeks.

Following the third dose of vaccines administered to 4 of 8 ponies in each of the vaccine groups (50, 15, 5, 1 μg HA/dose), 4, 3, 3 and 3 ponies respectively developed SRH antibody levels > 74 mm^2, consistent with protection.

Based on these results, more than three doses of aqueous vaccine of conventional potency (15 μg HA/dose; Wood *et al.* 1983*b*) would be required to stimulate protective levels of antibody in all ponies, but this study does not provide any

information on the duration of protective levels of antibody after three doses of vaccine.

This study also confirmed observations made previously that the severity of post-challenge clinical influenza correlated with pre-challenge circulating antibody. Numbers of temperature rises seen in the four pre-challenge antibody groups (< 4, 4–50, 51–100, > 100 mm^2) were inversely proportional to the antibody level, although no relationship could be demonstrated between antibody and duration of temperature responses. However, in this experiment the duration of febrile responses was short-lived in all groups, and biphasic responses recorded previously were not observed in these ponies. This observation may be a result of fewer ponies experiencing secondary bacterial infections.

In addition a clear relationship was demonstrated between antibody and numbers of ponies excreting virus, duration and amount of virus excreted and, as in the previous experiment, virus was recovered from some vaccinated ponies which did not exhibit any clinical signs of infection. Although antibody responses to the neuraminidase were demonstrated in these ponies (C. Gibson, personal communication), any relationship with protection has not been investigated.

Studies with influenza vaccine in man have also demonstrated that both serum antibody responses (Ennis *et al.* 1977; Nicholson *et al.* 1979) and protection (Goodeve, Jennings & Potter, 1983; Al-Khayatt, Jennings & Potter, 1984) are related to vaccine SRD potency. The 100% protective levels of SRH antibody for influenza H3N2, H1N1 and B infections were in the region of 90 mm^2. This is in good agreement with the equine system when differences in SRH assay sensitivity are taken into account.

Thus, while it is recognized that antibody produced locally in the respiratory tract may have an important role in immunity to influenza (Rouse & Ditchfield, 1970), as well as cell-mediated immune responses (Lin & Askonas, 1981), levels of circulating antibody to the haemagglutinin nevertheless provide a good measure of protection in ponies which have been immunized with inactivated virus vaccines. Similarly, intranasal challenge of ponies may be used as a challenge system to evaluate the efficacy of the H3N8 component of equine influenza vaccines on a comparative basis by measuring antibody responses, temperature responses and virus excretion in relation to pre-challenge antibody.

REFERENCES

Al-Khayatt, R., Jennings, R. & Potter, C. W. (1984). Interpretation of responses and protective levels of antibody against attenuated influenza A viruses using single radial haemolysis. *Journal of Hygiene* **92**, 301–312.

Ennis, F. A., Mayner, R. E., Barry, D. W., Manischewitz, J. E., Dunlop, R. C., Verbonitz, M. W., Bonzeman, F. M. & Schild, G. C. (1977). Correlation of laboratory studies with clinical responses to A/New Jersey influenza vaccines. *Journal of Infectious Diseases* **136**, Supplement, 397–406.

Goodeve, A. C., Jennings, R. & Potter, C. W. (1983). The use of the single radial haemolysis test for assessing antibody response and protective antibody levels in an influenza B vaccine study. *Journal of Biological Standardization* **11**, 289–296.

Lin, Y. L. & Askonas, B. A. (1981). Biological properties of an influenza A virus specific T-killer cell clone; inhibition of virus replication in vivo and induction of delayed type hypersensitivity reactions. *Journal of Experimental Medicine* **154**, 225–229.

MUMFORD, J. A., WOOD, J. M., SCOTT, A. J. M., FOLKERS, C. R. & SCHILD, G. C. (1983). Studies with inactivated equine influenza vaccines. 2. Protection against experimental infection with influenza virus A/equine/Newmarket/79 (H3N8). *Journal of Hygiene* **90**, 385–395.

NICHOLSON, K. G., TYRRELL, D. A. J., HARRISON, P., POTTER, C. W., JENNINGS, R., CLARK, A., SCHILD, G. C., WOOD, J. M., YETTS, R., SEAGROATT, V., HUGGINS, A. & ANDERSON, S. G. (1979). Clinical studies of monovalent inactivated whole virus and subunit A/USSR/77 (H1N1) vaccine: serological responses and clinical reactions. *Journal of Biological Standardization* **7**, 123–136.

ROUSE, B. T. & DITCHFIELD, W. J. B. (1970). The response of ponies to myxovirus influenza A-Equi-2. III. The protective effect of serum and nasal antibody against experimental challenge. *Research in Veterinary Science* **11**, 503–507.

WOOD, J. M., MUMFORD, J. A., FOLKERS, C. R., SCOTT, A. J. M. & SCHILD, G. C. (1983*a*). Studies with equine influenza vaccines. 1. Serological responses of ponies to graded doses of vaccine. *Journal of Hygiene* **90**, 371–384.

WOOD, J. M., SCHILD, G. C., FOLKERS, C., MUMFORD, J. A. & NEWMAN, R. W. (1983*b*). Standardization of inactivated equine influenza vaccines by single radial immunodiffusion. *Journal of Biological Standardization* **11**, 133–136.