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Relationship Between Onset of Puberty and Establishment of Persistent Infection with Equine Arteritis Virus in the Experimentally Infected Colt

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Summary

The relationship between stage of reproductive tract maturity and susceptibility to the experimental establishment of persistent infection with equine arteritis virus (EAV) was investigated in 21 prepubertal and 15 peripubertal colts. Five of six prepubertal colts inoculated intranasally remained infected in the reproductive tract from post-challenge day 28 to 93 and two of six from post-challenge day 120 to 180. No virus was detected in five of these animals killed on post-challenge day 210. Each of two peripubertal colts remained infected in the reproductive tract at post-challenge day 60 and one of nine was found to be persistently infected with EAV 15 months after challenge. These findings confirm that the virus can replicate in the reproductive tract of a significant proportion of colts for a variable period of time after clinical recovery in the absence of circulating concentrations of testosterone equivalent to those found in sexually mature stallions. Long-term persistent infection with EAV does not appear to occur in colts exposed to the virus before the onset of peripubertal development. We suggest that colts should be vaccinated at approximately 6 months of age, before peripubertal development but after the disappearance of maternally acquired antibodies.

Introduction

Equine viral arteritis is a contagious disease whose aetiology was defined in the early 1950s, when a virus was isolated from an outbreak of abortion and respiratory illness on a horse farm near Bucyrus, OH, U.S.A. (Doll et al., 1957a,b). The virus was named equine arteritis virus (EAV) and the disease equine viral arteritis (EVA), because of the distinctive vascular lesions found on histopathological examination of acutely infected cases (Doll et al., 1957a). EAV is classified in the genus Arterivirus, and was formerly considered a member of the non-arthropod-borne group of Togaviruses (Westaway et al., 1985). Recent studies have shown that the organization and expression of the EAV genome are similar to those of Coronavirus and Coronaviruses (Snijder et al., 1990). It has been suggested, therefore, that EAV may be taxonomically related to these two

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virus groups and belong to a Coronavirus-like superfamily (den Boon et al., 1991). The International Committee on Taxonomy of Viruses has recently removed the genus *Arterivirus* from the family Togaviridae and left it as a free-floating genus pending the assessment of current research (Pringle, 1992).

EVA was considered relatively unimportant until an epidemic occurred on a number of Thoroughbred breeding farms in Kentucky in 1984. Since then, the number of confirmed outbreaks has increased (Timoney and McCollum, 1991) due in part to heightened awareness. Investigation of the 1984 epidemic confirmed the existence of a carrier state in stallions, which appear to play a major role in maintaining and disseminating the virus months or years after clinical recovery (Timoney et al., 1986b). The rate of viral persistence in stallions varies from 25–65 per cent (Timoney et al., 1987; Neu et al., 1988; Timoney and McCollum, 1991; Timoney et al., 1992). Little information is available on the frequency of the carrier state in colts infected before puberty or during peripubertal development. The testosterone dependency of EAV output from persistently infected stallions has been reported by Little et al. (1992). The present study was undertaken in an attempt to establish persistent infection with EAV in prepubertal and peripubertal colts and, if successful, to determine the site of viral replication within the reproductive tract.

**Materials and Methods**

**Horses**

A total of 36 colts of mixed breeding and aged 5 to 12 months were purchased from a commercial source. An assessment of the maturity of the reproductive tract of each colt was based on age, serum testosterone concentration and ultrasonographic findings. The colts were assigned to either a prepubertal (nos 48–61, 64–70) or a peripubertal (nos 17–31) group. A total of 10 age-matched colts were used as controls (nos 111–118, 255, 257). All of the animals were confirmed as seronegative to EAV and equine infectious anaemia virus (EIAV) at the time of purchase and again immediately before inoculation with EAV. It should be noted that shortly before the start of this study, a *Streptococcus equi* infection spread through the group of peripubertal colts. All indications of infection had resolved before the study began.

**Cell Culture**

A continuous rabbit kidney cell line (RK-13; ATCC No. CCL 37) was used for attempted isolation and infectivity assay of EAV. Cell monolayers were grown in 25 cm² plastic tissue-culture flasks in 10 per cent modified Eagle’s minimal essential medium (EMEM), as described by Timoney et al. (1986b).

**Virus Inoculum**

The challenge inoculum was prepared from a strain of EAV similar to that used by Neu et al. (1988), isolated from Thoroughbred stallions during the 1984 epidemic in Kentucky (McCollum and Timoney, 1983).

**Animal Inoculation**

Colts in each experimental group were challenged intranaso-pharyngeally on "day 0" with 5 ml of a 10 per cent splenic tissue suspension administered by fenestrated nasal
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catheter. The inoculum contained $c. 10^4$ plaque forming units (PFU) per ml, both before challenge and after inoculation.

**Experimental Design**

The study consisted of two experiments. The first, on peripubertal colts, was made from March 1990 to June 1991. The second experiment, on prepubertal colts, was made from October 1990 to May 1991. Monitoring and sampling of each group of animals began one day before challenge and continued through the acute phase of infection to day 28 post-challenge. All colts were examined daily at c. 8.00 am and 4.00 pm for clinical signs, and rectal temperatures were taken. The following specimens were collected: citrated and clotted blood samples, nasopharyngeal swabs and, from peripubertal colts, semen.

The amount of peripubertal development of the colts was monitored monthly in both experimental groups by ultrasonographic examination and determination of serum testosterone concentrations (Holyoak, Little, Vernon, McCollum and Timoney, unpublished data).

In the first experiment (peripubertal colts) two animals were killed on days 7, 60, and 120 post-challenge. The remaining nine colts were held until it was feasible to collect semen samples for virus isolation; they were subsequently killed c. 15 months post-challenge.

In the second experiment (prepubertal colts) two animals were killed on days 7, 14, 28, 56, 93, 119, 150, and 180 post-challenge. The remaining five colts were killed on c. day 210.

All colts were killed by the intravenous injection of sodium pentobarbital solution (Beuthanasia, Schering) at a dosage of 1 ml per 10 lb body weight.

**Collection of Specimens**

Rectal temperatures and clinical signs were monitored as described above. Nasopharyngeal swabs and citrated blood samples were obtained 24 h before challenge and on days 2, 5, 7, 9, 12, 14 and 16 from the peripubertal group; 24 h before challenge and on days 3, 5, 7, 9, 11, 13, 15, 17, 21 and 28 from the prepubertal group; and just before euthanasia in both groups. Clotted blood samples were collected 24 h before challenge and on days 7, 28, 42 and 56, and approximately twice a month thereafter.

Semen was collected from the peripubertal colts in experiment 1 for attempted virus isolation and to assess pubertal status. Puberty was confirmed by the presence of 50 million spermatozoa per ejaculate with $\geq 10$ per cent motility. Semen samples were obtained with the help of a Missouri style artificial vagina (Nasco Co., Ft Atkinson, WI, U.S.A.) and ovariohysterectomized mares, treated with oestradiol cypionate to induce oestrous behaviour. Collection attempts were initiated during the second week in May, 1991, approximately 59 weeks post-challenge, and continued at weekly intervals until five collections were obtained from each colt, when the experiment was terminated.

Tissues and body fluids were collected aseptically as soon after death as possible. The following samples were obtained from each prepubertal and peripubertal colt: (fluids) pericardial, peritoneal, thoracic, urine; (reproductive tract tissues) testes, vasa deferentia, ampullae, vesicular glands, prostatic lobes, bulbourethral glands and proximal and distal urethra; (non-reproductive tissues) lung, liver, spleen, kidneys, ileum, and inguinal, mesenteric, colonic, splenic and bronchial lymph nodes. The sequence of specimen collection from the reproductive tract was as follows: bulbourethral glands, prostate and vesicular glands (care being taken to avoid perforating the pelvic urethra), the ampullae, deferent ducts, epididymides and testes. Specimens for virus isolation were placed immediately on wet ice in a vacuum flask for transport to the laboratory, where they were stored at $-20^\circ$C. Tissues for light
microscopy were fixed in 10 per cent neutral buffered formalin at the time of collection.

Virus Isolation

Nasopharyngeal swabs, citrated blood samples, tissues and body fluids were processed for virus isolation as previously described (McCollum et al., 1971) except that monolayer cell cultures of RK-13 cells (Timoney et al., 1986b) were used. Samples of gel-free semen were subjected to sonication and centrifugation before inoculation as described by Timoney et al. (1987).

Virus Identification

Virus isolates were confirmed as EAV by a one-way serum neutralization plaque-reduction assay. Serial decimal dilutions of selected isolates were made in EMEM plus 2 per cent fetal bovine serum containing 10 per cent guinea-pig complement (Pel-Freeze Biologicals; Rogers, AR, U.S.A.). A heat-inactivated reference equine serum containing antibodies against the challenge strain of EAV was used.

Serological Response

Serum neutralizing antibodies to EAV were assayed by means of a micro-neutralization test and monolayer cultures of RK-13 cells in the presence of 10 per cent guinea-pig complement (Senne et al., 1985).

Ultrasonographic Examination

To assess acute disease-induced changes within the reproductive tract, all of the colts in the study were palpated per rectum, followed by ultrasonographic examination of the accessory sex glands 24 h before challenge and on days 7, 14 and 28 post-challenge. Ultrasonographic examinations were performed with a Corometrics Aloka 500V (Corometrics Medical Systems, Wallingford, CT, U.S.A.) fitted with a 7.5 MHz linear array transducer.

Semen Examination

A small volume of gel-free semen was taken from each sample immediately after collection, placed on a pre-warmed microscope slide, and examined microscopically for spermatozoa and percentage motility. Additional small volumes were taken to determine sperm concentration by means of a haemocytometer, and the percentage of abnormal spermatozoa by counting 200 cells per ejaculate in smears fixed in 40 per cent ethyl alcohol and examined by differential interference-contrast microscopy.

Results

Clinical Findings

All of the 36 prepubertal and peripubertal colts experimentally infected with EAV developed clinical signs. The colts became febrile, with similar temperature curves for both groups, maximum temperatures being recorded c. 6 to 10 days post-challenge. Maximal rectal temperatures in each group ranged from 39.6°C to 41°C (mean, 40.4°C). Apart from fever, the clinical signs varied, each animal showing at least one of the following: serous to mucopurulent
ocular or nasal discharge, oedema of the limbs, scrotum or prepuce, scleral injection, conjunctivitis, icterus, cough, diarrhoea, stiff gait, lethargy, inappetence, and depression. The most common sign was serous or mucopurulent nasal discharge, followed by conjunctivitis, limb oedema and lethargy. The clinical signs persisted for 2 to 18 days (mean, 10). There were no major differences in clinical signs between the two groups of colts.

Ultrasonographic examination showed that, before challenge, all of the 21 prepubertal colts had an ampulla diameter of 3 mm which increased to 4 mm by post-challenge day 14. In all but one of these colts the ampulla diameter returned to 3 mm by post-challenge day 28. In several colts a variable degree of perirectal oedema was detectable ultrasonographically. No other abnormality was detected ultrasonographically in either group of colts.

Virological Findings

Nasopharyngeal Swabs. EAV was isolated from both groups of colts from post-challenge, days c. 2 to 17. All prepubertal colts had ceased shedding virus in the respiratory tract by day 21.

Buffy Coats. EAV was isolated on post-challenge days 2 to 16 (peripubertal group) and day 17 (prepubertal group). From the peripubertal group, virus was recovered up to day 16, when sampling ended. From the prepubertal group, virus was not isolated on day 28, which marked the end of the sampling. Combining the results of the two experimental groups, the duration of viraemia ranged from 5 to 15 days, with an average of 8·5 days (SD = 2·9). The average duration of viraemia in the prepubertal group was 7·9 days (SD = 1·42), with a range of 5 to 11 days, while that for the peripubertal group was 9·1 days (SD = 3·04), with a range of 5 to 15 days. These figures were compiled excluding data on the six colts killed during the acute phase.

Tissues and Body Fluids. EAV isolation from the reproductive tract tissues of the prepubertal and peripubertal colts is shown in Tables 1 and 2 respectively. Isolation results for the non-reproductive tract tissues of both experimental groups, from days 7 to day 60 post-challenge, are given in Table 3.

Virus was isolated from all tissues and body fluids collected from the four colts (nos 17, 19, 54 and 65) killed on post-challenge day 7. Infectivity titres ranged from $10^2$ to $10^6$ PFU per ml of a 10 per cent tissue suspension or ml of body fluid (Tables 1-3). EAV had completely cleared from various sites in the two prepubertal colts (nos 67 and 70) killed on day 14. However, although virus was no longer detectable in the body fluids, lung or liver, it could still be isolated from most of the reproductive tract sites as well as from all of the lymph nodes sampled. By post-challenge day 28, a few of the lymph nodes contained concentrations of virus, but none was detectable in the testes or bulbourethral glands of the two colts sampled (nos 60 and 69; Table 1). EAV was isolated from various tissues throughout the reproductive tract in the four colts killed on day 60 (peripubertal colts nos 28 and 31, and prepubertal colts nos 52 and 58), but none could be detected in the lymph nodes, testes or bulbourethral glands. Virus was isolated from both of the prepubertal colts
**Table 1**

Virus concentration in reproductive tract tissues from prepubertal colts on days 7 to 210 after experimental infection with equine arteritis virus

| Colt no. | Day | Testis | Epididymis | Vas deferens | Ampulla | Vesicular gland | Prostate | Bulbourethral gland |
|---------|-----|--------|------------|--------------|---------|----------------|----------|---------------------|
|         |     | Ht     | Bdy        | Ht           | Bdy     | Ht             | Bdy      | Ht                  |
| 54      | 7   | 5-1    | 5-2        | 4-7          | 3-2     | 2-9            | 2-0      | 4-2                 |
| 65      | 7   | 4-0    | 7-2        | 3-1          | 2-9     | 2-9            | 2-0      | 4-1                 |
| 67      | 14  | 2-1    | 2-0        | 3-7          | 3-7     | 4-2            | 4-1      | -                   |
| 70      | 14  | 2-2    | 2-1        | 2-1          | 2-1*    | 2-1*           | 2-0      | 2-0*                |
| 60      | 28  | 2-0*   | 1-2*       | 2-9          | 2-5     | 2-1*           | 2-5      | 1-7                 |
| 89      | 28  | 2-0*   | 2-2*       | 2-0*         | 2-7*    | 2-8*           | 2-0      | -                   |
| 52      | 56  | 1-0†     | 1-1†   | -            | -       | 1-2†          | -        | -                   |
| 58      | 56  | 1-2*   | 3-2*       | 3-5          | 3-2     | 2-4            | 3-1      | 1-1                 |
| 50      | 92  | -      | -          | -            | -       | -              | -        | -                   |
| 59      | 93  | 1-1*   | 3-2*       | 3-5          | 3-2     | 2-3*           | 2-6*     | -                   |
| 49, 64  | 120 | -      | -          | -            | -       | -              | -        | -                   |
| 51      | 150 | 2-1    | 1-5†       | 4-5          | 3-3     | 3-4            | 3-5      | -                   |
| 53      | 150 | -      | -          | -            | -       | -              | -        | -                   |
| 55      | 180 | 1-0    | 1-2        | 4-2          | 3-1     | 3-0            | 3-2      | 2-0                 |
| 56      | 180 | -      | -          | -            | -       | -              | -        | -                   |
| 48, 57  | 210 | -      | -          | -            | -       | -              | -        | -                   |
| 61, 66, 68 | 210 | -      | -          | -            | -       | -              | -        | -                   |

**Ht** = head, **Bdy** = body, **Ht** = tail, **Prox.** = proximal, **Dist.** = distal.

Virus titre expressed as log_{10} PFU per ml of 10 per cent tissue suspension.

* Virus isolated from right side only.
† Virus isolated from left side only.
- = Negative for virus.
Table 2
Virus concentration in reproductive tract tissues from prepubertal colts on days 7 to 450 after experimental infection with equine arteritis virus

| Colt no. | Day | testis | epididymis | vas deferens | ampulla | vesiculargland | prostate | bulbourethralgland | semen* |
|---------|-----|--------|------------|-------------|---------|----------------|----------|------------------|--------|
|         |     |        | Hdad | Bdy | T1 | Prox. | Dist. | Prox. | Dist. | Prox. | Dist. | Prox. | Dist. | Prox. | Dist. | Prox. | Dist. | Prox. | Dist. | Prox. | Dist. |
| 17      | 7   | 5:5    | 5:4   | 5:3  | 4:5  | 4:6  | 5:6  | 5:1  | 4:3  | 5:1  | N/A   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 19      | 7   | 4:5    | 6:1   | 5:2  | 5:3  | 4:8  | 4:5  | 4:6  | 5:1  | 4:7  | 5:1  | N/A   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 28      | 60  |        | 4:0   | 5:4  | 4:6  | 2:4  |      |      |      |      |      | N/A   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 31      | 60  |        | 4:1   | 5:0  | 1:8  |      |      |      |      |      |      |      | N/A   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 18      | 120 |        | 5:0   | 4:1  | 5:0  |      |      |      |      |      |      |      | N/A   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 20      | 120 |        | 4:1   |      |      |      |      |      |      |      |      |      |      | N/A   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 21      | 450 |        | 4:0   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 22      | 450 |        | 4:0   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 23      | 450 |        | 4:0   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 24      | 450 |        | 4:0   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 25      | 450 |        | 4:0   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 26      | 450 |        | 4:0   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 27      | 450 |        | 4:0   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 29      | 450 |        | 4:0   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| 30      | 450 |        | 4:0   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |

Hd = head, Bdy = body, T1 = tail, Prox. = proximal, Dist. = distal.
Virus titres expressed as log_{10} PFU per ml of 10 per cent tissue suspension.
* Average infectivity titre of five consecutive weekly semen collections.
- = Negative for virus.
N/A = not attempted.
### Table 3

Virus concentration in non-reproductive tract tissues from prepubertal and peripubertal colts on days 7 to 60 after experimental infection with equine arteritis virus

| Colt no. | Day | Lung | Lvo. | Spl. | Kid. | Ing. Ln. | Mes. Ln. | Col. Ln. | Spl. Ln. | Brn. Ln. | Perit. Fl. | Peric. Fl. | Pleur. Fl. | Urine |
|----------|-----|------|------|------|------|----------|----------|----------|----------|----------|------------|------------|------------|-------|
| 54       | 7   | 3.3  | 3.2  | 3.1  | 3.1  | 6.2      | 5.2      | 5.9      | 5.1      | 5.1      | 3.6        | 3.4        | 3.3        | 2.2   |
| 65       | 7   | 3.4  | 4.2  | 4.1  | 4.2  | 6.2      | 5.3      | 6.1      | 5.7      | 6.3      | 4.1        | 4.1        | 3.6        | 2.5   |
| 17*      | 7   | 4.3  | 2.8  | 5.2  | 3.3  | 5.8      | 6.7      | 6.2      | 6.3      | 5.6      | 3.5        | 4.3        | 4.2        | 1.5   |
| 19*      | 7   | 4.1  | 3.2  | 4.3  | 3.7  | 5.3      | 6.3      | 5.3      | 6.2      | 6.5      | 4.2        | 4.3        | 4.2        | 2.1   |
| 67       | 14  | —    | —    | 2.2  | 3.2  | 3.1      | 2.3      | 3.3      | 3.5      | 3.2      | —          | —          | —          | —     |
| 70       | 14  | —    | —    | 1.1  | 2.5  | 2.4      | 2.1      | 2.8      | 3.1      | 2.2      | —          | —          | —          | —     |
| 60       | 28  | —    | —    | 1.1  | —    | —        | 1.2      | —        | —        | 1.1      | —          | —          | —          | —     |
| 69       | 28  | —    | —    | 1.2  | 1.9  | —        | 2.4      | 1.1      | —        | —        | —          | —          | —          | —     |
| 52       | 60  | —    | —    | —    | —    | —        | —        | —        | —        | —        | —          | —          | —          | —     |
| 58       | 60  | —    | —    | —    | —    | —        | —        | —        | —        | —        | —          | —          | —          | —     |
| 28*      | 60  | —    | —    | —    | —    | —        | —        | —        | —        | —        | —          | —          | —          | —     |
| 31*      | 60  | —    | —    | —    | —    | —        | —        | —        | —        | —        | —          | —          | —          | —     |

Virus titres expressed in log₅ PFU per ml of 10 per cent tissue suspension or per ml of fluid.

— = Negative for equine arteritis virus.

Liv. = Liver, Spl. = spleen, Kid. = kidney, Ing. Ln. = inguinal lymph node, Mes. Ln. = mesenteric lymph node, Col. Ln. = colonic lymph node, Spl. Ln. = splenic lymph node, Brn. Ln. = bronchial lymph node, Perit. Fl. = peritoneal fluid, Peric. Fl. = pericardial fluid, Pleur. Fl. = pleural fluid.

* = Peripubertal colts. The other eight animals were prepubertal.
killed on day 56. In prepubertal colt no. 52, virus was detected only in the
distal portions of the left epididymis and in the distal extremity of the left
ampulla of the vas deferens. In contrast, virus was isolated from many sites
throughout the reproductive tract of colt 58, from the epididymis to the
prostate gland. EVA was isolated from reproductive tract tissues extending
from the tail of the epididymis to the prostate gland of both peripubertal colts
(nos 28 and 31) killed on day 63. It was also recovered from one of the two
prepubertal colts (nos 50 and 59) killed on day 93. In colt 59 however, virus
was no longer detectable in samples of the prostate, vesicular glands, testes or
bulbourethral glands. It was isolated only from the right side of the repro-
ductive tract, including the epididymis, vas deferens and the ampulla of the
vas deferens (Table 1). Of the four animals killed on day 120 (two prepubertal
colts [nos 49 and 64] and two peripubertal colts [nos 18, 20]) none yielded EAV.
EAV was isolated, however, from one (no. 51) of the two prepubertal colts (nos
51 and 53) killed on day 150. It was recovered from the epididymis to the
ampulla of this animal, but not from any tissues distal to the ampulla. Tissues
with the highest infectivity were the tail of the epididymis and the ampulla,
with titres of $10^5$ and $3 \times 10^4$ PFU per ml, respectively. These findings were
remarkably consistent in the persistently infected prepubertal colts. Similarly,
virus was also isolated from one of the two prepubertal colts (nos 55 and 56)
killed on day 180. In colt 55, virus was recovered from the epididymis to the
prostate of both right and left sides of the reproductive tract. A total of four of
the eight prepubertal colts remained infected with EAV from the end of the
acute phase to day 180. EAV was not detected, however, in any of the colts
(nos 48, 57, 61, 66 and 68) killed at the conclusion (days 209 and 210) of the
prepubertal study.

Of the nine colts in the peripubertal group that were maintained for c. 15
months after challenge, one (no. 29) had virus in its semen (Table 2). All five
collections from this colt yielded virus (titre c. $10^3$ PFU per ml) and the semen
and ampullae were also virus-positive at the conclusion of the study. EAV was
isolated from both the right and left ampullae ($10^4$ PFU per ml) but from no
other site sampled (Table 2). The other eight colts had no virus in their semen
or in tissues and other body fluids collected at time of necropsy.

Infectivity Assays

The concentration of EAV in the tissues sampled during the acute phase of the
disease was highest in the lymph nodes, ranging from $10^5$ to $10^6$ PFU per ml
(Table 3). In reproductive tract tissues virus concentrations ranged from $10^3$ to
$10^5$ PFU per ml. The highest virus titres in colts killed after the acute phase
were obtained from sites in the reproductive tract distal to the testes ($10$ to $10^4$
PFU per ml; Tables 1 and 2). The tail of the epididymis and ampulla of the vas
deferens consistently had the highest infectivity titres in the prepubertal colts
killed from days 60 to 180.

A significant number of randomly selected virus isolates were confirmed as
EAV in a one-way neutralization assay.
Serological Responses

Serum neutralizing (SN) antibodies were detected in samples collected from the second week after inoculation to the completion of each experiment in all colts of both groups. Individual SN antibody responses from the prepubertal and peripubertal colts are summarized in Tables 4 and 5, respectively. In the prepubertal groups, titres recorded on day 14 ranged from 4 to 64 (mean, 16). With one exception (no. 55) all of these colts attained a maximal SN titre by day 60. Maximal titres attained in the prepubertal colts ranged from 64 to 256. The maximal mean titre (128) was recorded in this group of colts on days 28 and 60. The data from colts killed on or before day 28 were not included in these calculations.

The pattern of SN antibody response in the peripubertal colts (Table 5) differed from that in the prepubertal colts (Table 4). The initial response was similar, with titres ranging from 8 to 64 and a mean titre of 32 recorded on day 14. Whereas the prepubertal colts attained maximal titres near day 60, only one peripubertal colt (no. 18) had reached a titre of 128 by day 60 and one more (no. 24) by day 90. Colt 24 then appeared to experience an anamnestic response with its titre increasing sharply to $\geq 512$ at day 300. Titres in all of the other peripubertal colts increased very gradually up to $c.$ day 180, after which all of the nine remaining colts reached their maximum SN antibody titres from days 210 to 330. The maximal titres attained by these colts varied from 128 to $\geq 512$. The maximal mean titre of 256 was recorded for this group of colts on day 270.

Discussion

Preliminary observations on a limited number of young horses indicated that the frequency of persistent EAV infection in sexually immature colts with antibodies to the virus was either low or nil (McCollum and Timoney, unpublished data). This is in contrast to the reported carrier rate of between 25 and 62 per cent in naturally and experimentally infected stallions (Timoney et al., 1987; Neu et al., 1988) and up to 65 per cent in a recent survey of naturally infected stallions (Timoney et al., 1992). Observations suggesting that the shedding of infectious virus in the semen of persistently infected stallions is testosterone-dependent have also been reported (Little et al., 1992). Generally, young colts less than 10 months of age do not synthesize pubertal amounts of testosterone (Naden et al., 1990). The present study was undertaken to investigate prepubertal and peripubertal colts experimentally challenged with EAV in respect of their tendency to become persistently infected, and to investigate a possible relationship between any such tendency and the degree of reproductive tract maturity at time of viral infection.

All of the challenged prepubertal and peripubertal colts became infected with EAV and developed clinical signs of EVA similar to those reported previously (Doll et al., 1957b; McCollum and Timoney, 1985; Timoney, 1985; Neu, 1988).
Table 4
Serum neutralizing antibody titres in 21 prepubertal colts after experimental infection with equine arteritis virus

| Days before (minus) or after inoculation | SN titres in colts no. |
|----------------------------------------|-----------------------|
|                                       | 48 | 49 | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 64 | 65 | 66 | 67 | 68 | 69 | 70 |
| -30                                    | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 |
| -7                                     | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 |
| 7                                      | 4  | 4  | 4  | 4  | 4  | 4  | 4  | 4  | 4  | 4  | 4  | 4  | 4  | 4  | 4  | 4  | 4  | 4  | 4  | 4  | 4  | 4  |
| 14                                     | 8  | 4  | 4  | 4  | 8  | 4  | 16 | ±4 | 4  | ±4 | 4  | 64 | 4  | 8  | 8  | 4  | 32 | ±4 | 4  | 16 | 64 |
| 21                                     | 32 | 16 | 4  | 32 | 8  | 32 | 32 | 32 | 4  | 16 | 128 | 8  | 8  | 8  | 8  | 32 | 128 |
| 28                                     | 64 | 32 | 128 | 64 | 128 | 32 | 16 | 32 | 128 | 64 | 256 | 64 | 64 | 256 | 64 | 256 | 32 | 256 |
| 60                                     | 32 | 16 | 256 | 64 | 64 | 32 | 64 | 64 | 64 | 32 | 256 | 32 | 64 | 64 | 128 | 256 |
| 90                                     | 64 | 32 | 128 | 16 | 32 | 64 | 32 | 128 | 128 | 32 | 64 | 8  | 32 | 128 |
| 120                                    | 64 | 32 | 32 | 64 | 64 | 32 | 128 | 32 | 64 | 16 | 64 |
| 150                                    | 64 | 32 | 64 | 128 | 32 | 64 | 32 | 64 | 16 | 128 |
| 180                                    | 32 | 128 | 32 | 64 | 32 | 32 | 64 |
| 210                                    | 64 | 64 | 64 | 16 | 32 |
Table 5
Serum neutralizing antibody titres in 15 peripubertal colts after experimental infection with equine arteritis virus

| Days before or after inoculation | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 |
|---------------------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| -60                             | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 |
| -21                             | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 |
| -7                              | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 |
| 7                               | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 | <4 |
| 14                              | 32 | 16 | 32 | 32 | 32 | 8  | 32 | 16 | 32 | 32 | 32 | 64 | 32 | 16 | 64 |
| 28                              | 64 | 32 | 32 | 32 | 16 | 16 | 32 | 8  | 32 | 32 | 64 | 8  | 32 | 16 | 16 |
| 60                              | 128| 32 | 32 | 32 | 32 | 64 | 32 | 32 | 32 | 32 | 64 | 32 |
| 90                              | 64 | 32 | 64 | 32 | 32 | 64 | 128| 64 | 16 | 32 | 16 | 16 |
| 120                             | 32 | 32 | 32 | 64 | 64 | 64 | 32 | 32 | 32 | 64 | 64 |
| 150                             | 64 | 64 | 32 | 32 | 64 | 128| 16 | 32 | 64 |
| 180                             | 32 | 128| 128| 32 | 256| 32 | 128| 32 | 128|
| 210                             | 128| 128| 128| 128| 512| 32 | 256| 64 | 256|
| 240                             | 64 | 64 | 512| 256| 512| 128| 256| 128| 512|
| 270                             | 64 | 64 | 512| 256| 512| 128| 256| 128| 512|
| 300                             | 32 | 128| 128| 128| 512| 512| 64 | 128| 64 |
| 330                             | 32 | 128| 128| 128| 512| 512| 64 | 128| 64 |
| 360                             | 32 | 32 | 64 | 256| 512| 32 | 128| 64 | 128|
| 390                             | 32 | 32 | 64 | 256| 512| 32 | 128| 64 | 128|
| 450                             | 32 | 64 | 64 | 128| 256| 16 | 128| 64 | 64 |

EAV was isolated from nasopharyngeal and buffy coat samples from all of the colts during the acute phase of the infection. Viraemia ranged in duration from 5 to 15 days (mean, 8.5). These findings are similar to those found in experimentally infected stallions (Neu, 1988).

SN antibodies were detected in all of the colts from the second week after inoculation to the completion of each experiment. Maximal titres attained in both groups of colts were similar to those reported previously (McCollum, 1970; Neu, 1988). The slow rise of titres observed in the peripubertal colts suggested continued antigenic stimulation during the period of the experiment. The source of this stimulation and why it took so long for maximal titres to be attained is unclear. Viruses such as lactate dehydrogenase virus, a single stranded RNA virus, are immunosuppressive and induce a delayed and weak neutralizing antibody response in mice (Inada and Mims, 1986). Lymphocytic choriomeningitis virus, also an RNA virus which persistently infects mice, infects lymphocytes and alters the immune responsiveness of infected cells (Oldstone, 1989a). EAV has not been reported to be immunosuppressive.

Testosterone treatment suppresses antibody response in experimental mice (Carlsten et al., 1989; Vendramini et al., 1991). The effects of sex steroids on immune response have been the subject of a review by Schuurs and Verheul (1990), which suggested that normal physiological concentrations of circulating testosterone have immunosuppressive activity. The testosterone concentrations in the reproductive tract of the peripubertal colts may have been
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responsible for the drawn-out antibody response, which contrasted with the normal response found in the prepubertal colts, which had low serum testosterone concentrations (unpublished data). Another possible explanation for the variability in response may be that the peripubertal colts were immunosuppressed due to an earlier *Streptococcus equi* infection (J. F. Timoney, personal communication) which spread through this group of animals shortly before the start of the study. Whatever the cause, the delayed rise in antibody titres to EAV appeared to have little effect on the eventual percentage of colts persistently infected with the virus, with the exception of colt 29, which would have been under maximal immunosuppressive effects due to increasing testosterone levels. This colt entered the peripubertal phase of development before the study began (unpublished data).

Virus was isolated from all tissues and body fluids collected from the four colts killed on day 7. By day 14, EAV had been eliminated from various sites. It was no longer detected in body fluids or from lung and liver tissue. Virus was still isolated from most of the reproductive tract tissues, however, as well as from all of the lymph nodes sampled. The sites of viral replication were further restricted by day 28, in that EAV was only detected in reproductive tract tissues from the epididymis to the prostate, and in a few of the lymph nodes. The findings for the first 2 weeks after challenge were very similar to those previously reported (McCollum et al., 1971; Fukunaga et al., 1981). After day 60, virus was isolated most commonly from the tail of the epididymis and the ampulla. The highest viral titres were also obtained from these sites. The eventual restriction of viral replication sites to the reproductive tract has also been reported in experimentally infected stallions (Neu, 1988).

From the findings of the present study, it is concluded that EAV can replicate for a considerable period within the reproductive tract tissues of both prepubertal and peripubertal colts. Stallions persistently infected for 8 to c. 21 weeks or longer after challenge are considered to have become chronic carriers (Timoney et al., 1987; Neu et al., 1988). The results of these earlier experimental studies, as well as various field observations (Timoney et al., 1986b; Timoney and McCollum, 1991), are consistent with the findings recorded up to 21 weeks after challenge in the present study in colts. Beyond 21 weeks, however, there are considerable differences between the behaviour of the virus in the stallion and in the sexually immature colt.

The long-term virus persistence in both groups of colts was far less than that found in experimentally (Neu et al., 1988) and naturally infected stallions (Timoney et al., 1986a; Timoney et al., 1992). None of five prepubertal colts killed on day 210 had detectable infectious virus, and only one (no. 29) of nine peripubertal colts killed 15 months after challenge was persistently infected with EAV. This was the only colt of the final group of nine that had any peripubertal development at the time of experimental challenge. Thus this colt had higher concentrations of serum testosterone and greater accessory sex gland dimensions from the outset than any other colt in the final group of nine peripubertal colts. Virus was isolated from each of five semen samples collected ante-mortem from colt 29, and at necropsy exclusively from its ampullae.
It can be concluded that EAV replication is localized to the reproductive tract soon after the acute phase of infection in both pre- and peripubertal colts. The sites of replication are restricted even further by day 60 post-challenge. It appears that, in colts exposed to EAV while undergoing peripubertal development, virus is harboured almost exclusively in the ampulla of the vas deferens, a very similar finding to that in persistently infected stallions (Timoney and McCollum, unpublished data). Conversely, those colts from both experimental groups that were infected before the onset of peripubertal development did not maintain a long-term persistent infection. This is in sharp contrast to the 25 to 65 per cent carrier rate reported in experimentally or naturally infected seropositive stallions (Timoney et al., 1987; Neu et al., 1988; Timoney and McCollum, 1991; Timoney et al., 1992).

The establishment of persistent EAV infection is dependent on factors not yet completely defined. It is likely that persistence is associated with continual re-infection of susceptible tissues within the reproductive tract, and possibly with the evolution of antigenic variants of the virus (Murphy et al., 1988; Murphy et al., 1993). Constant shedding of virus in the semen of the mature stallion would appear to be testosterone dependent, according to Little et al. (1992), who found that there was either viral clearance or greatly decreased viral output after the surgical castration of naturally infected carrier stallions. Conversely, castrated stallions given testosterone supplementation to normal physiological levels did not show a significant drop in viral output in the semen or elimination of infectious virus from the reproductive tract tissues. These results are supported by the evidence of immunosuppressive activity of testosterone (Carlsten et al., 1989; Schuurs and Verheul, 1990; Vendramini et al., 1991). High circulating testosterone concentrations are clearly not necessary, however, for viral replication and persistence of intermediate duration in the prepubertal colt, but appear to be essential for long-term persistence.

The strain of EAV, infective dose and route of inoculation used in the present study, were as used by Neu et al. (1988). In the present study, intermediate persistent infection of EAV among prepubertal and peripubertal colts occurred in the absence of pubertal levels of testosterone. This was probably due to specific viral receptors (Mims, 1989) in the tissues during both the prepubertal and adult phases of development. The persistence in young colts suggests that, at least for this strain of EAV, there is a strong tropism for certain reproductive tract tissues and a tendency for intermediate persistence, in the absence of testosterone or other specific factors that may be present in the adult.

Persistence of EAV in the reproductive tract of the colt, in the face of moderate to high titres of homologous SN antibodies, suggests that humoral immunological interference with viral replication within these tissues is minimal or non-existent. Oldstone (1989b) indicated that effectiveness of the humoral immune response can be blunted by changes in viral epitope expression, such as down-regulation of viral glycoprotein production to levels much lower than those found in the acute phase of infection. It was also proposed that in certain infections the antibodies themselves initiate persistent infections by binding viral antigens presented on host cell surfaces, thus
removing or masking these antigens without slowing the progress of infection (Fujinami and Oldstone, 1984; Oldstone, 1989b). This antigen masking would then render the infected cell resistant to T cell-mediated immune responses as well as possible antibody dependent cellular cytotoxicity. Whether or not EAV utilizes any of these mechanisms in the establishment of persistent infections is not known.

The present study and various field observations (Timoney and McCollum, 1991; Timoney and McCollum, unpublished data), suggest a carrier phase of intermediate duration. Five of six prepubertal colts challenged intranasally remained infected in the reproductive tract from post-challenge day 28 to 93, and two of six from post-challenge day 120 to 180. None of the five remaining colts were still infected one month later. Each of two peripubertal colts remained infected in the reproductive tract at post-challenge day 60. These data from both groups suggest the existence of an intermediate carrier state, possibly lasting up to 6 months. A carrier state of intermediate duration is supported by data obtained from naturally infected stallions (Timoney and McCollum, unpublished data). A significant reduction in persistent infection was seen 6–7 months after initial exposure of this particular group of stallions to EAV. Because the study of Neu (1988) was terminated at day 148 post-challenge, an intermediate-term carrier state of the range seen in the current study and in the cited field data may have gone undetected.

In the present study, there appeared to be a correlation between the onset of peripubertal development and long-term persistence of EAV in the reproductive tract. If age at the time of inoculation is disregarded, and serum testosterone concentrations and the stage of reproductive tract maturity are the main criteria, none of the colts infected before undergoing peripubertal development remained long-term (>6 months) persistent carriers of EAV. This suggests that the onset of peripubertal changes, and not age alone, is the principal factor governing persistent EAV infection. It would therefore appear that weanling colts are at low risk of developing long-term persistent infection, whereas yearling colts may be at low to high risk, depending on the state of reproductive tract maturity at the time of exposure.

The principal findings of this study are that EAV continues to replicate in tissues of the reproductive tract after the resolution of the clinical phase of infection; a carrier state of intermediate duration may be established, which can last for up to 6 months after infection and which occurs in the absence of circulating concentrations of testosterone equivalent to those found in the sexually mature stallion; and finally, termination of viral persistence in prepubertally challenged colts may be associated with the onset of puberty, but the precise mechanism has not been determined. Prepubertal colts are thus unlikely to become long-term carriers of EAV. This finding may have broad implications in controlling the establishment of a carrier state in the stallion. If colts were vaccinated at approximately 6 months of age, before peripubertal development but after the disappearance of maternally acquired antibodies, it is probable that the incidence of EVA and inapparent EAV infection would eventually decline because of a reduction in the population of carrier stallions.
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