Reproduction and sera embryotoxicity after immunization of monkeys with the laminin peptides YIGSR, RGD, and IKVAV

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ABSTRACT Monkeys with excellent reproductive histories were immunized with the laminin peptides YIGSR, RGD, IKVAV, and YD, a control sequence with no known biological function. Sera from the YIGSR-immunized monkey became toxic, causing neural tube defects in whole rat embryo cultures, and this monkey experienced fetal loss after immunization. Sera from the RGD-immunized monkey also became embryotoxic in culture after immunization, but this monkey appeared to become infertile as she failed to initiate a pregnancy for at least 2 years after immunization. In contrast, embryos cultured on sera from the IKVAV- or YD-immunized monkeys were predominantly normal and both monkeys completed successful pregnancies. Antibody levels to the respective peptides or to laminin were not predictive of embryotoxicity, but antibody binding to homogenized yolk sacs as well as to yolk sacs of cultured embryos was associated with sera embryotoxicity and reproductive outcomes in vivo. These observations suggested that the laminin sequences YIGSR and RGD may play a role in immune-mediated reproductive failure by reacting directly with embryonic tissue and could provide a basis for identifying individuals at risk for both spontaneous abortion and infertility.

Immune dysfunction has been implicated in recurrent pregnancy loss (1). For example, isolated T cells from recurrent aborters were found to secrete embryotoxic cytokines in response to trophoblast cells (2), while a direct role of autoantibodies in fetal loss has been implicated for women with autoimmune diseases, such as systemic lupus erythematosus (3) and herpes gestationis (4). Anti-phospholipid autoantibodies particularly have been associated with spontaneous abortions (5), but their presence alone could not predict poor pregnancy outcomes (6). Therefore, there has been a need to identify specific autoantibodies responsible for reproductive failure and to establish their mechanisms of pathogenicity (7).

Anti-laminin autoantibodies were detected in the sera of some monkeys with histories of reproductive failure, and sera from these monkeys caused neural tube defects in cultures of whole rat embryos (8). When monkeys with excellent reproductive histories were immunized with intact murine laminin, their sera became embryotoxic, again causing neural tube defects in rat embryo cultures, and these monkeys subsequently failed to reproduce (9). When pregnant mice were injected with heterologous anti-laminin antibodies, they spontaneously aborted (10). However, sera from laminin-immunized rats were not consistently embryotoxic and sera anti-laminin antibody levels were not predictive of embryotoxicity (11). These toxic and nontoxic sera were found to recognize different epitopes on Western blots of enzymedigested laminin, suggesting that antibodies to only certain laminin epitopes were responsible for embryotoxicity. Further support for such embryotoxic laminin epitopes was obtained by using anti-laminin monoclonal antibodies, as each antibody was observed to have not only a unique dose–response relationship to embryotoxicity but also caused specific embryo abnormalities (12). More recently, using two laminin-immunized monkeys, we found that the severity of the cultured embryo responses to their sera was related to the levels of antibody binding to yolk sacs and was associated with the recognition of different laminin epitopes (13).

Laminin, a 900-kDa extracellular matrix protein, has been found to be multifunctional with diverse activities associated with specific sequences (14). For example, the YIGSR sequence was found to be involved with cell attachment, tumor metastasis, and angiogenesis, while the laminin RGD sequence was found to influence cell attachment and neurite outgrowth (15). The sequence IKVAV was found to be involved with neurite outgrowth, tumor metastasis, platelet adhesion, and T-cell binding (14, 16). It should be noted that most of these studies were done using in vivo systems.

In the present study, monkeys with excellent reproductive histories were immunized with laminin peptides. With sera from these monkeys, the toxicities of the anti-peptide antibodies were tested in whole rat embryo cultures, and the monkeys were mated to observe possible effects of immunization on reproduction. From these studies, two laminin domains were identified that caused both toxicity to cultured rat embryos and reproductive failure in the monkeys.

MATERIALS AND METHODS

Monkeys. The pigtail monkeys (Macaca nemestrina) were maintained at the University of Washington Primate Center and were selected because of their excellent reproductive histories and for the ability of their sera to support normal cultured rat embryo development before immunization. Each monkey was immunized with 0.5 mg of the appropriate peptide (Table 1) and given a booster injection 6 wk later with 0.25 mg (9). Breeding was initiated 2 wk after the booster injection. For the IKVAV peptide, a second monkey was immunized as the first developed a physical vaginal blockage and was unable to breed. Blood samples were collected from the femoral vein and processed for whole embryo culture (9).

Embryo Culture. Embryos at the headfold stage of development (9.5 days of gestation) were isolated from CD strain rats and cultured for 48 hr in 90% serum/10% water (9). Where indicated, l-methionine supplementation (48 μg/ml) replaced water. Embryos were examined and photographed at the end of the culture period and could be divided into four

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morphological types (see Fig. 3). Embryo protein content was
determined by the Lowry method (17).

**Yolk Sac Homogenates.** Yolk sacs were isolated from 12-day
embryos (CD strain) and placed in phosphate-buffered saline
(PBS; 0.13 M NaCl/0.15 M Na₂HPO₄, pH 7.5), sonicated with
three 10-s pulses, and centrifuged for 2 min at 13,500 × g; the
supernatant was stored at −20°C.

**ELISA.** Antibody levels were determined as described (9)
using the method of Voller et al. (18). Polystyrene microtiter
plates were coated with either 1 μg of the YIGSR or YD
peptide, 0.1 μg of the IKVAV or RGD peptide, 10 μg of mouse
tumor laminin, or 0.1 μg of protein from homogenized yolk
sacs. The sera were serially diluted (log₂) in 0.05% Tween in
phosphate-buffered saline (PBS-T) and incubated for 30 min
at room temperature. Preimmune sera from each monkey were
mixed to a row of wells to serve as background binding for
determinations on each plate. Horseradish peroxidase-linked
goat anti-monkey IgG diluted 1:200 in PBS-T was used to
detect bound antibodies. Antibody binding was visualized
using as substrate 0.18 mg of 2,2'-azinobis(3-ethylbenzthiozolino
sulfonic acid) (ABTS) in 0.05 M citrate buffer containing
hydrogen peroxide. Changes in the optical density over a 4-h
incubation period were measured and antibody levels were
expressed as the reciprocal of the dilution factor corresponding
to 50% of maximal optical density values of the difference
between readings at time 0 and 4 min.

**Antibody Avidity.** The avidities of the antibodies to their
respective peptides and to laminin were determined with
monkey sera drawn 16 wk after the initial immunization (19).
Horseradish peroxidase-linked goat anti-monkey IgG was used
with the ABTS substrate and the avidity index was defined as
the molar concentration of ammonium thiocyanate required
to reduce the optical density by 50% relative to the wells without
ammonium thiocyanate.

**Isolation of IgG.** The IgG fraction was isolated using an
Econo-Pac 10 DG desalting column (Bio-Rad) followed by an
Affi-Gel Blue column (Bio-Rad) and finally dialysis against
Tyrode’s solution and concentration with a Centriprep-30 con-
centrator (Amicon) (20).

**Immunofluorescence.** Indirect immunofluorescence was
performed on 10-day-old rat embryos as described (8) with
the following modifications. Sera from the immunized
monkeys were diluted 1:10 in PBS-T, while the fluorescein isothio-
cyanate-linked goat anti-monkey IgG antibodies (Organon Tech-
nika–Cappel) were diluted 1:40. Sections were visualized with a
Zeiss Axioplan microscope with epillumination.

To localize antibody binding during culture, rat embryos
were cultured for 3 h on rat serum containing 11 mg of isolated
IgG fractions per ml from the peptide-immunized monkeys.
The embryo sections were prepared as described (8) and
incubated with a 1:500 dilution of fluorescein isothiocyanate-
linked goat anti-monkey IgG prior to examination (Zeiss).

**Uptake Studies.** To examine the effect on yolk sac function
of embryos cultured on sera from the peptide-immunized
monkeys, the uptake of [methyl-14C]methionine (specific
activity, 47 mCi/mmol; 1 Ci = 37 GBq; ICN) and [U-14C]sucrose
(specific activity, 8.0 mCi/ml) was measured after 21 h of
culture as described (13). The methionine uptake by the yolk
sacs was corrected for the protein content of the yolk sacs, cpm
detected in the media, and concentration of free methionine
in the sera (21). The sera methionine concentrations were 8.0,
7.1, and 6.9 μg/ml for the YIGSR-, RGD-, and YD-
immunized monkeys, respectively. The sucrose uptake by
the yolk sacs was corrected for the protein content of the yolk sac
and cpm in the media.

**RESULTS**

**Antibody Analyses.** Before immunization, sera from all
monkeys were tested by ELISA and found to be negative for
anti-laminin antibodies when compared with sera from a
bovine serum albumin-immunized monkey. After immuniza-
tion, the sera from these monkeys were tested against their
respective peptides and against laminin in ELISA. The
YIGSR-immunized monkey produced low levels of antibodies
to YIGSR that peaked at ~30 wk (Fig. 1A). Sera from this
monkey also had antibodies that bound to laminin and this
response was maintained throughout the first year of immu-
numization (Fig. 2A). The sera from the RGD-immunized mon-
key reached peak antibody levels on the 8th wk (2 wk after
the booster immunizations) to both the RGD peptide (Fig. 1B)
and laminin (Fig. 2B), although levels to both decreased appreciably after 16 wk but remained detectable for at least
a year after immunization. As the sequence RGD has been
found to be present in other proteins (22), sera from the
RGD-immunized monkey were tested against other basement
membrane proteins and were found to bind to both fibronectin
collagen type IV. The IKVAV-immunized monkey also
produced low levels of anti-IKVAV antibodies that peaked on
the 8th wk (Fig. 1C) but, in contrast to the other immunized
monkeys, antibody binding to laminin was not detected (data
not shown). A second monkey immunized with IKVAV gave
comparable responses to both the IKVAV peptide and lamin-
in. Sera from the monkey immunized with the control peptide
(YD) had peak levels of anti-YD antibodies on wk 4 and 8 after
immunization (Fig. 1D) and were also found to have high levels
of antibody binding to laminin on wk 2 and 8 after immu-
numization (Fig. 2C).

![Fig. 1. Sera antibody levels to peptides after immunization of monkeys with YIGSR (A), RGD (B), IKVAV (C), and control peptide YD (D). Both preimmune and postimmune sera from each monkey were serially diluted (log₂). Preimmune sera were taken as background and levels were expressed as the reciprocal of the dilution factor corresponding to 50% of maximal optical density values of the differences between optical densities of preimmune and postimmune sera at 0 and 4 min.](image-url)
somite pairs). This embryotoxicity could be overcome by the addition of L-methionine (49 µg/ml) to the culture media, as had been observed previously for sera from laminin-immunized monkeys (13). Sera from the RGD-immunized monkey were also embryotoxic (Table 3) but, unlike the YIGSR-immunized monkey, the embryo responses were not as severe and the embryos had predominantly closed neural tubes (Table 3). One year after immunization, serum from this monkey was no longer embryotoxic, which corresponded to a reduction in circulating antibody levels (Fig. 1). For the IKVAV-immunized monkey, the first two sera samples (drawn at wk 2 and 4 after immunization) were embryotoxic with morphological types C and D, and one additional sample drawn at 20 wk was also toxic. Similarly, the first two sera samples tested in embryo cultures with the YD monkey were also toxic and of the D morphological type while one additional toxic sample was observed at wk 16. Correcting for the first two postimmune samples (assuming they were caused by the immunization) would give the following abnormality frequencies: YIGSR, 88%; RGD, 50%; IKVAV, 20%; YD, 21%. These 20–21% abnormality frequencies were comparable to that observed in an earlier study involving a monkey immunized with bovine serum albumin (13).

**Antibody Binding to Embryonic Tissues.** Using indirect immunofluorescence on uncultured 10-day-old rat embryos, sera antibodies from both the YIGSR- and RGD-immunized monkeys were found to bind to the cells of the visceral yolk sac endoderm and to the acellular Reichert’s membrane. In contrast, sera antibodies from the YD-immunized monkey bound uniformly to all embryonic tissues. Sera antibodies from the IKVAV-immunized monkey failed to bind to tissues of the embryo proper or to the yolk sac.

When sera from the peptide-immunized monkeys were tested against homogenized yolk sacs by ELISA, the sera antibodies from the YIGSR-immunized monkeys had significantly greater binding (optical density, 26.0 ± 5.6) than sera antibodies from, in decreasing order, the RGD- (8.3 ± 2.4), YD- (5.0 ± 0.6), and IKVAV- (2.0 ± 0.5) immunized monkeys. To examine the binding of these antibodies to the yolk sacs during culture, embryos were exposed to control rat sera containing 11 mg of isolated IgG fractions from each monkey for 3 h. By using direct immunofluorescence on frozen embryo sections, antibodies from the YIGSR- and RGD-immunized monkeys were found to bind exclusively to the cell surfaces of the visceral yolk sac endoderm (Fig. 4 A and B) and, as predicted by the ELISA results, the anti-YIGSR antibodies appeared to have the greatest immunoreactivity. Antibodies from the other two monkeys (IKVAV and YD) exhibited little or no binding to the yolk sacs from cultured embryos.

**Yolk Sac Uptake.** After 21 h of culture, the uptake of [14C]methionine by yolk sacs from embryos cultured during 1- and 3-h exposures was lower in the presence of sera from the YIGSR- and RGD-immunized monkeys than from the YD-immunized monkey (Table 4). This was observed in two separate experiments. For the uptake of [14C]sucrose, again sera from the YIGSR-immunized monkey had the lowest level of uptake relative to sera from the YD-immunized monkey but sera from the RGD-immunized monkey did not appear to inhibit sucrose uptake (Table 4).

**Reproductive Trials.** Before immunization, the YIGSR-immunized monkey had completed five successful pregnancies of six initiated, whereas after immunization this monkey initiated two pregnancies that ended in fetal loss—the first after 130 days and the second after 50 days (normal mean gestation, 170 days). Before immunization, the RGD-immunized monkey completed five successful pregnancies of five initiated but after immunization she was unable to become pregnant in spite of repeated trials and the use of at least three different males. In normal monkeys after the initiation of pregnancy, progesterone levels were found to increase to

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**Fig. 2.** Sera antibody levels to murine laminin after immunization of monkeys with YIGSR (A), RGD (B), and control peptide YD (C). Both preimmune and postimmune sera from each monkey were serially diluted (log2). Preimmune sera were taken as background and levels were expressed as the reciprocal of the dilution factor corresponding to 50% of maximal optical density values of the differences between optical densities of preimmune and postimmune sera at 0 and 4 min.

The antibody avidities to their respective peptide and laminin were compared using ammonium thiocyanate with 16 wk postimmune sera. Antibodies from the YIGSR-, RGD-, and YD-immunized monkeys had high avidities to their respective peptides compared with the avidity of antibodies from the IKVAV monkey (Table 2). Against laminin, the avidities of the antibodies from both the YIGSR- and RGD-immunized monkeys were significantly greater than those from both the IKVAV- and YD-immunized monkeys (Table 2).

**Embryo Cultures.** Although the YIGSR-immunized monkey had low antibody levels to YIGSR, sera samples from this monkey were the most toxic to cultured rat embryos when compared with sera from the other three monkeys and remained toxic over 2 years. The embryos were predominantly of the abnormal morphological type D (Table 3, Fig. 3D) with open neural tubes (exencephaly), absence of eye development (anhphthalmia), fusion of the posterior and anterior neural folds (dorsifusion), reduced growth (decreased protein accumulations), and limited differentiation (decreased number of

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**Table 2.** Avidities of antibodies from peptide-immunized monkeys to their respective peptide or laminin

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Avidity ± SE (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YIGSR</td>
<td>2.95 ± 0.31 (3)</td>
</tr>
<tr>
<td>RGD</td>
<td>2.21 ± 0.01 (3)</td>
</tr>
<tr>
<td>IKVAV</td>
<td>1.18 ± 0.07 (3)</td>
</tr>
<tr>
<td>YD</td>
<td>3.98 ± 1.0 (3)</td>
</tr>
</tbody>
</table>

Avidity values are given in molar concentration of ammonium thiocyanate required to reduce optical density by 50%. Different superscripts (* and **) indicate that avidities between the four peptide-immunized monkeys were significantly different at \( P < 0.05 \) using GLM and PDIFF functions from the statistical analysis system (23).
Table 3. Cultured rat embryo responses to monkey sera before and after peptide immunization

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Immune status</th>
<th>Abnormal/total embryos (%)</th>
<th>Morphological type</th>
<th>Protein accumulation, µg per embryo ± SE (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A  B  C  D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YIGSR</td>
<td>Preimmune</td>
<td>0/4  (0)</td>
<td>23.3 ± 1.4 (4)</td>
<td>172 ± 2 (2)</td>
</tr>
<tr>
<td></td>
<td>Postimmune</td>
<td>27/30 (90)*</td>
<td>13.3 ± 0.8 (30)*</td>
<td>68 ± 11 (8)*</td>
</tr>
<tr>
<td>RGD</td>
<td>Preimmune</td>
<td>1/6  (17)</td>
<td>21.2 ± 1.5 (6)</td>
<td>170 ± 6 (2)</td>
</tr>
<tr>
<td></td>
<td>Postimmune</td>
<td>9/16 (64)*</td>
<td>16.5 ± 1.0 (14)</td>
<td>109 ± 21 (8)</td>
</tr>
<tr>
<td>IKVAV</td>
<td>Preimmune</td>
<td>1/6  (17)</td>
<td>19.0 ± 0.3 (6)</td>
<td>127 ± 38 (3)</td>
</tr>
<tr>
<td></td>
<td>Postimmune</td>
<td>10/34 (29)</td>
<td>18.8 ± 0.5 (34)</td>
<td>110 ± 7 (22)</td>
</tr>
<tr>
<td>YD</td>
<td>Preimmune</td>
<td>0/4  (0)</td>
<td>23.0 ± 1.2 (4)</td>
<td>187 ± 3 (2)</td>
</tr>
<tr>
<td></td>
<td>Postimmune</td>
<td>7/18 (38)</td>
<td>18.6 ± 1.1 (18)</td>
<td>119 ± 16 (10)</td>
</tr>
<tr>
<td>Combined</td>
<td>Preimmune</td>
<td>2/20 (10)</td>
<td>21.2 ± 0.6 (20)</td>
<td>160 ± 14 (9)</td>
</tr>
</tbody>
</table>

Morphological types: A, closed neural tubes, expanded optic vesicles, and completed ventral curvature; B, closed neural tubes and incomplete ventral curvature; C, open neural tube (exencephaly), failure of the optic vesicle to expand (anophthalmia), and complete ventral curvature; D, exencephaly, anophthalmia, and fusion of posterior to anterior neural tubes (dorsifusion).

*Numbers of abnormal embryos from postimmune sera differed significantly from combined preimmune sera at P < 0.01 by Fisher's exact test.

†Protein accumulation or somite pairs differed significantly between combined preimmune sera and postimmune sera at P < 0.001 by Mann–Whitney test (34).

15–20 ng per ml of blood (24). However, the progesterone levels for the RGD-immunized monkey after mating ranged between 2 and 9 ng/ml, which was equivalent to her normal cycle, suggesting that fertilization had not occurred. In contrast to the YIGSR- and RGD-immunized monkeys, the IKVAV-(11/11 successful pregnancies before immunization) and YD-(10/10 successful pregnancies) immunized monkeys completed pregnancies (1 each) with normal offspring.

**DISCUSSION**

Previously, sera from laminin-immunized monkeys were found to cause neural tube defects in cultured rat embryos and these monkeys no longer reproduced successfully (9). In the present study, sera from the YIGSR-immunized monkey caused similar defects in cultured rat embryos and this monkey experienced two spontaneous abortions. Sera from the monkey immunized with the peptide RGD also caused embryotoxicity, but the abnormalities were less severe and persisted for a shorter period than those observed with sera from the YIGSR-immunized monkey. The RGD-immunized monkey appeared to become infertile. Embryos cultured on sera from the monkeys immunized with the peptide IKVAV or the control peptide YD were predominantly normal and these monkeys completed pregnancies. Thus, in agreement with previous studies, cultured embryo responses to sera were directly related to reproductive performance in the intact animal (25).

As previously observed (11, 12), a relationship between anti-laminin antibody levels against laminin or the peptides and embryotoxicity was not apparent, but both serum embryotoxicity and monkey reproductive outcome were related to the level of antibody binding to homogenized yolk sacs as well as to yolk sacs during embryo culture. Therefore, embryotoxicity of anti-laminin antibodies appeared related to the exposure of specific laminin peptide epitopes on the cell surfaces of the visceral yolk sac endoderm. This binding could play a critical role in anti-laminin antibody embryotoxicity as it was found to reduce nutrient flow into the yolk sacs and to the embryos. Previously, this reduction in nutrient flow was associated with decreased numbers of microvilli found on the cell surfaces of visceral yolk sac endoderm (13). It was interesting to note that anti-laminin antibodies as well as the YIGSR peptide could alter the structure of F-actin in Sertoli cells (26), as this protein has been found to be a structural element of microvilli (27). In the YIGSR-immunized monkey,

**Fig. 3.** Whole rat embryos after 48 h of culture with extraembryonic membranes removed. (A) Normal embryos with closed neural tubes, expanded optic vesicles, and completed ventral curvature. (B) Embryos with closed neural tubes and incomplete ventral curvature. (C) Embryos with open neural tubes (exencephaly), failure of the optic vesicles to expand (anophthalmia), and complete ventral curvature. (D) Embryos with exencephaly, anophthalmia, and fusion of posterior to anterior neural tubes (dorsifusion). cnt, Closed neural tube; ont, open neural tube; ov, optic vesicle; df, dorsifusion. (×5.)

**Fig. 4.** Direct immunofluorescence of frozen embryos cultured for 3 h on rat sera containing isolated IgG fractions from YIGSR-(A), RGD-(B), IKVAV-(C), and YD-(D) immunized monkeys. Only antibodies from the YIGSR- and RGD-immunized monkeys were observed to bind to the visceral yolk sac endoderm (end). (×200.)
Table 4. Uptake of [14C]methionine and [14C]glucose by yolk sacs from embryos cultured from bodies also by port women suffering with studies RGD antibodies were chloride-induced glomerulonephritis gas infertile subjects laminin studies antibodies that have could. Furthermore, we cultured of the culture medium. and medium, of the RGD culture sacs. Finally, we detected anti-YIGSR antibodies in sera of some Brown Norway rats (Rattus norvegicus) with mercuric chloride-induced glomerulonephritis in strains of mice susceptible to systemic lupus erythematosus and in monkeys with histories of reproductive failure. In addition, we found anti-YIGSR antibodies in sera of some individuals with Chagas disease, women with systemic lupus erythematosus, and women suffering from recurrent spontaneous abortions. Anti-RGD antibodies were not detected in these studies, but infertile subjects have not been tested. Although epidemiological studies would be needed to establish the importance of these laminin sequences to reproductive disorders in general, these studies could provide the basis for treatments such as oral tolerization (31) or anti-idiotypic antibody therapy (32) that have been used to treat autoimmune diseases. Alternatively, dietary supplements with the amino acid methionine could provide a less invasive approach to treatment. We have found that adding methionine to whole embryo cultures overcame neural tube defects caused by anti-laminin and anti-YIGSR antibodies. Furthermore, in preliminary trials, dietary methionine supplements were found to improve reproductive outcomes in some monkeys (33) and women with histories of poor reproduction (23). Hopefully, the approach used in this study with whole embryo cultures could be applied to the identification of other antibodies responsible for reproductive failure and to the development of treatments based on their mechanisms of embryotoxicity.

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<table>
<thead>
<tr>
<th>Peptide</th>
<th>Uptake of [14C]methionine*</th>
<th>Uptake of [14C]glucose†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>3 h</td>
</tr>
<tr>
<td>YIGSR</td>
<td>363</td>
<td>810</td>
</tr>
<tr>
<td>RGD</td>
<td>348</td>
<td>459</td>
</tr>
<tr>
<td>YD</td>
<td>542</td>
<td>993</td>
</tr>
</tbody>
</table>

* cpm corrected for yolk sac protein, level of radioactivity in the culture medium, and concentration of free methionine in the sera.
† cpm corrected for yolk sac protein and level of radioactivity in the culture medium.