Preconception Vaccination with a Glycoprotein B (gB) DNA Vaccine Protects against Cytomegalovirus (CMV) Transmission in the Guinea Pig Model of Congenital CMV Infection

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DNA vaccines expressing the guinea pig cytomegalovirus (GPCMV) homologs of the glycoprotein B (gB) and UL83 proteins were evaluated for protection against congenital GPCMV infection. After 4 doses of DNA administered by epidermal (gene gun) route, all guinea pigs developed enzyme-linked immunosorbent assay (ELISA) antibody and, for gB-vaccine recipients, neutralizing antibody. Dams were challenged with $1 \times 10^4$ plaque-forming units of GPCMV in the third trimester. Preconception vaccination with gB did not decrease overall pup mortality, although, within the gB-vaccine group, pup mortality was lower among dams with high ELISA responses. Preconception maternal vaccination with gB vaccine significantly reduced congenital transmission in liveborn pups. In contrast, UL83 vaccine had no significant effect on pup mortality or vertical transmission of GPCMV. Virus load was significantly lower in infected pups born to gB- and UL83-vaccinated dams than in infected pups born to control dams. These data support the concept that subunit gB vaccination may be useful in protecting against CMV-induced disease.

Human cytomegalovirus (HCMV) infection, although generally asymptomatic in normal hosts, can result in severe disease in immunocompromised hosts, including newborn infants [1]. Acquisition of infection in utero can produce particularly devastating consequences, including neurodevelopmental sequelae and sensorineural deafness [2, 3]. Congenital HCMV infection occurs in 1%–2% of all pregnancies, complicating an estimated 40,000 pregnancies annually in the United States [4]. Since preconception maternal immunity to HCMV provides some degree of protection against vertical transmission of infection [5, 6], there has been considerable interest in development of CMV vaccines. HCMV vaccines capable of protecting newborns from the neurodevelopmental sequelae caused by congenital infection would be highly cost-effective, and development of such vaccines was recently identified as a “level 1” (most cost-effective category) priority by the Institute of Medicine [7].

Since the correlates of the maternal immune response to HCMV that are critical to protection of the fetus are unknown, it is unclear which vaccine strategy would be optimal. A variety of HCMV vaccines have been evaluated in clinical trials [8–11]. These have included live attenuated vaccines, as well as subunit and vectored vaccines, targeting the HCMV proteins that appear to be most important in protective immunity. Protein targets include the major envelope glycoprotein gB (gpUL55) and the tegument phosphoprotein pp65 (ppUL83) [12]. The majority of virus-neutralizing antibody responses after HCMV infection recognize gB [13], whereas pp65 elicits the majority of the CD8+ cytotoxic T lymphocyte (CTL) response to infection [14, 15]. Recombinant HCMV gB expressed in CHO cells is safe and well tolerated and elicits...
virus-neutralizing antibody responses after intramuscular (im) administration [16, 17]. Both gB and UL83 have been expressed in a recombinant canarypox (ALVAC) system and have undergone limited evaluation in phase 1 studies; these vaccines, too, are immunogenic and well tolerated [18–21]. However, at present, there has not been an evaluation of the efficacy of any HCMV vaccine for prevention of congenital infection.

Ideally, preclinical evaluations of the immunogenicity and efficacy of HCMV vaccines would be performed in animal models of congenital infection. Unfortunately, the species specificity of CMV precludes evaluation of HCMV vaccines in animals and necessitates the study of animal CMVs [22]. In contrast to the CMVs of most small animals, guinea pig CMV (GPCMV) is unique in its ability to cross the placenta, causing infection in utero [23]. This feature makes the GPCMV model useful for evaluation of vaccine strategies. Previous studies have indicated that preconception vaccination of guinea pigs, using native GPCMV proteins administered with potent adjuvants, provides protection against GPCMV-induced pup mortality and infection, after viral challenge of pregnant guinea pigs [24–26]. However, the lack of detailed molecular characterization of GPCMV proteins has, at present, precluded any studies in this model using cloned, recombinant expression technologies. In recent studies, the GPCMV homologs of gB and UL83 (GP83) have been cloned [27, 28], and the immunogenicity of candidate subunit vaccines based on these genes, expressed as DNA plasmid vaccines, has been characterized [29]. The advantage of using DNA vaccine for such studies is the ease with which such vaccines can be generated and the usefulness they offer as “proof of concept” for the protective roles of specific viral genes in vaccine/challenge studies. The present study was undertaken to test the protective efficacy of DNA vaccines that target the GPCMV homologs of gB and UL83 (GP83), administered by epidermal (gene gun) route, in the guinea pig model of congenital CMV infection.

MATERIALS AND METHODS

Animal studies. Young female and proven-breeder male Hartley guinea pigs were obtained from Harlan Laboratories. Inbred adult strain-2 guinea pigs were purchased from the Children’s Hospital Research Foundation (Cincinnati, OH). Guinea pigs were confirmed to be GPCMV seronegative by ELISA before vaccination. Animals were housed under conditions approved by the American Association of Accreditation of Laboratory Animal Care, in accordance with institutional animal-use committee policies.

Virus and cells. GPCMV (strain no. 22122; ATC VR682) was propagated on guinea pig fibroblast lung cells (GPL; ATCC CCL 158) and was maintained in F-12 medium supplemented with 10% fetal calf serum (HyClone Laboratories), 10,000 IU/L penicillin, 10 mg/L streptomycin (Gibco-BRL), and 7.5% NaHCO3, (Gibco-BRL). Salivary gland–passaged stocks (SG virus) were prepared by sequential passage in strain-2 guinea pigs, as described elsewhere [30].

Recombinant plasmids, DNA vaccine preparation, and experimental design. The cloning details used for generation of gB and GP83 plasmid constructs are described elsewhere [27–29]. In brief, the GPCMV gB homolog was expressed in a truncated, secreted form, spanning amino acid residues 1–692, in a plasmid designated pKTS 404. The GP83 protein was expressed as a full-length construct (aa 1–565) in a plasmid designated pKTS 437. Both plasmids use the HCMV major immediate-early promotor and a polyadenylation signal derived from the bovine growth hormone gene (pCDNA 3.0; Invitrogen). Plasmid DNA was purified by use of Qiagen column and was conjugated to gold particles, as described elsewhere [29].

To examine the protective efficacy of the DNA vaccines, young female Hartley guinea pigs were vaccinated with a series of 4 epidermal inoculations of gB (pKTS 404) or GP83 (pKTS 437) plasmid at monthly intervals, as described elsewhere [29]. For each vaccination, animals were inoculated with 6 doses (2 μg) of plasmid conjugated to 0.5 mg of gold “carrier” particles. Approximately 30 days after the fourth vaccination, blood was obtained for immunogenicity analyses, and the animals were placed with breeder males and were examined weekly by palpation for evidence of pregnancy. In the third trimester of pregnancy, animals were inoculated subcutaneously with 1 × 103 pfu of GPCMV and were observed daily until delivery to determine the outcome of pregnancy.

ELISA, neutralization assays, and radioimmunoprecipitation (RIP)–PAGE analyses. Antibody responses in vaccinated animals were monitored by ELISA. ELISA was performed using GPCMV antigen, as described elsewhere [31]. ELISA titers were defined as the reciprocal of the highest dilution that produced an absorbance of at least 0.10 and twice the absorbance against control antigen. Neutralization assays were performed by use of serum samples from guinea pigs vaccinated with pKTS 404, as described elsewhere [29], except that 5% rabbit serum was used as a source of exogenous complement. Neutralization assays were performed by use of an isolate of GPCMV tagged with green fluorescent protein, and plaques were enumerated by fluorescence microscopy [32]. Neutralization titers were defined as the highest dilution of serum that resulted in a ≥50% reduction in plaques, compared with that in control (preimmune) serum samples. Serum samples from vaccinated animals were further examined by RIP-PAGE for immunoreactivity with GPCMV proteins. GPL cells were inoculated with GPCMV (MOI, ∼5 pfu/cell), and, at 96 h after infection, cells were incubated in media supplemented with 35S-cysteine and me-
thionine (35S-Translabel; ICN Radiochemicals) at a specific activity of 50 μCi/mL, for 4 h. Proteins were immunoprecipitated with antibody from vaccinated guinea pigs (volume, 20 μL), as well as hyperimmune, polyclonal anti-GPCMV antibody and *Staphylococcus aureus* protein A, and were subjected to SDS-PAGE and autoradiography, as described elsewhere [28].

**Viral culture and quantitative competitive PCR (qcPCR) analyses.** Liveborn pups were killed within 72 h of delivery, and tissue homogenates (10% wt/vol) of liver and spleen were prepared for viral culture on GPL cells. DNA from homogenates was extracted by use of the Qiagen QIAamp DNA mini kit DNA extraction system, according to the manufacturer’s specifications. Eluted DNA (1% of sample) was subjected to qcPCR analysis, as described elsewhere [33]. In brief, the primer pair UL83F6 (5′-CGACGCAGCGATGACGAAAAC-3′) and UL83B11 (5′-TCCTCGGTCTCAACGAAAGGTC-3′) amplifies a 225-bp region, corresponding to Asp402 through Ser473 of GP83. This plasmid was modified by engineering a 68-bp internal deletion. The resultant clone served as an internal standard (IS) for qcPCR. A standard curve was generated by measuring the ratio of relative signal intensity of amplification products on ethidium bromide–stained gels for increasing amounts of full-length plasmid with IS. The signal intensity of the experimental standard was compared with this standard curve to quantify the total copy number (GPCMV genome equivalents) per milligrams of tissue extracted.

**Statistical analyses.** Incidence data were compared by use of Fisher’s exact test. Continuous variables were compared by use of Student’s *t* test. All comparisons were 2-tailed.

**RESULTS**

**Immune response to DNA vaccination.** Previous analyses of the immunogenicity of GPCMV gB and GP83 expression plasmids indicated that 4 doses of vaccine are required for optimal immunogenicity by epidermal inoculation [29]. ELISA indicated that all animals (with gB and with GP83) seroconverted to GPCMV antigen after 4 doses of plasmid inoculation (figure 1). As observed elsewhere, ELISA titers were consistently higher after vaccination with gB plasmid, compared with those after vaccination with GP83 plasmid [29]. The mean ELISA titer in gB-vaccinated animals was 3.3 log_{10}, compared with 1.8 log_{10} in GP83-vaccinated animals (figure 1; *P* < .0001). In serum from gB-vaccinated guinea pigs that became pregnant, complement-dependent neutralizing titers were determined by plaque-reduction methods. Neutralizing titers ranged from 1:80 to 1:1280, with a mean neutralizing titer of 2.55 log_{10} (figure 1B).

**Immunoprecipitation analyses using serum samples from vaccinated guinea pigs.** To confirm that vaccination with recombinant gB plasmid induced antibodies capable of immunoprecipitating the native gB complex, RIP-PAGE analyses were performed (figure 2). All vaccinated animals made antibodies capable of immunoprecipitating species of ~150 kDa (representing the uncleaved intracellular gB precursor protein), ~90 kDa (representing the amino-terminal moiety), and ~58 kDa (representing the carboxy-terminal moiety) from 35S-labeled, GPCMV-inoculated tissue culture lysates.

**Outcomes of pregnancy in vaccine/challenge study.** To assess the protective efficacy of GPCMV subunit DNA vaccines against congenital CMV infection, a challenge experiment was performed after animals were bred. A total of 17 guinea pigs were vaccinated with gB plasmid, and 14 were vaccinated with GP83 plasmid. Among gB-vaccinated animals, 13 became pregnant and underwent GPCMV challenge; 1 was excluded from the analysis because she gave birth <7 days after challenge. Among GP83-vaccinated animals, 11 became pregnant and un-
Vaccination with gB DNA against CMV

Figure 2. Immunoprecipitation analysis of serum from gB–vaccinated animals. Serum (volume, 20 μL) from vaccinated guinea pigs and Staphylococcus aureus protein A were used to immunoprecipitate 35S-labeled lysates from guinea pig cytomegalovirus (GPCMV)–infected guinea pig fibroblast lung cells. Representative results observed using serum samples from 7 gB-vaccinated animals (including serum samples from dams with both high-mortality and low-mortality litters) are indicated (lanes 1–7). All animals engendered antibody capable of immunoprecipitating the ∼90 and ∼58 kDa subunits of the gB complex. In addition, an ∼150 kDa polypeptide representing the intracellular gB precursor was identified (arrows). Immunoprecipitation with preimmune serum samples did not demonstrate any GPCMV polypeptides (data not shown). Position of molecular weight markers is indicated. Immunoprecipitation profile of polypeptides immunoreactive with polyclonal anti-GPCMV antisera is also indicated (lane P).

Table 1. Pup mortality after maternal inoculation with guinea pig cytomegalovirus.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dams</th>
<th>Pups</th>
<th>Dead pups</th>
<th>Mortality, %</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>39</td>
<td>13</td>
<td>33</td>
</tr>
<tr>
<td>UL83 vaccine</td>
<td>11</td>
<td>38</td>
<td>13</td>
<td>34*</td>
</tr>
<tr>
<td>gB vaccine, ELISA titer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;3.4 log_{10}</td>
<td>4</td>
<td>13</td>
<td>0</td>
<td>0b</td>
</tr>
<tr>
<td>≤3.4 log_{10}</td>
<td>8</td>
<td>28</td>
<td>14</td>
<td>50</td>
</tr>
<tr>
<td>Overall</td>
<td>12</td>
<td>41</td>
<td>14</td>
<td>34*</td>
</tr>
</tbody>
</table>

Data are no. of mice, unless otherwise noted. gB, glycoprotein B.

* P not significant vs. control

b P < .005, vs. gB ELISA ≤ 3.4 log_{10}; P < .0001, vs. control

derwent GPCMV challenge; 1 was excluded from the analysis because she gave birth <7 days after challenge. Pregnancy was established in animals vaccinated with gB, GP83, or negative control plasmid. After challenge in the third trimester, pup mortality was compared. Outcomes of pregnancy were compared among animals for which there was a window of at least 7 days between GPCMV challenge and delivery of pups (table 1). For control dams (n = 10 litters), pup mortality was 33% (13/39). In pups born to gB-vaccinated (n = 12 litters) and GP83-vaccinated (n = 11 litters) dams, pup mortality was similar: 34% (14/41) for the gB-vaccine group and 34% (13/38) for the GP83-vaccine group. Although overall mortality in the gB-vaccine group was similar to that in the control group, significant differences in pup mortality were observed within the gB-vaccine group as a function of the magnitude of the ELISA titer. In litters born to dams with an ELISA titer <3.4 log_{10} (n = 8), pup mortality was 50% (14/28), a rate not statistically significantly different from that of the negative control group. In contrast, in pups born to dams with a mean log_{10} ELISA titer >3.4 log_{10} (n = 4), no pup mortality was observed (0/13; P < .005 vs. dams with ≤3.4 log_{10} ELISA titer; P < .0001 vs. negative controls) (table 1).

Effect of DNA vaccination on congenital GPCMV infection rates. To analyze the effect of DNA vaccination on congenital GPCMV infection, liveborn pups were killed within 72 h of delivery and then dissected, and organs (liver and spleen) were homogenized for tissue culture and PCR detection of viral genome. Among liveborn pups, preconception vaccination with gB vaccine, but not UL83 vaccine, resulted in a significant decrease in the incidence of congenital CMV infection (table 2). Of 26 liveborn pups in the control group, 17 had evidence of GPCMV infection by culture, and an additional 3 pups with negative cultures had viral DNA demonstrated by PCR, for a total congenital infection rate of 77% (20/26). In contrast, only 7 (26%) of 27 pups in the gB-vaccine group had evidence of GPCMV infection by viral culture, and an additional 4 pups had viral DNA demonstrated by PCR, for a total congenital infection rate of 41% (11/27; P < .005 vs. control group). Of 25 pups born to dams vaccinated with GP83, 13 had evidence of GPCMV infection by viral culture, and an additional 4 pups had viral DNA demonstrated by PCR, for a total congenital infection rate of 68% (17/25; P is not significant vs. control).

Analysis of virus load in GPCMV–infected pups. To fur-
Other evaluate the effect of DNA vaccine on congenital infection, virus loads were compared among infected pups in the gB- and GP83-vaccine groups and the control group by qPCR (table 2). Mean virus loads in infected pups born to gB- and GP83-vaccinated dams were significantly lower than those in control pups. In infected pups born to gB-vaccinated dams, the mean virus load in the liver was 1.8 log_{10} genomes/mg and in the spleen was 1.3 log_{10} genomes/mg. In infected pups born to GP83-vaccinated dams, the mean virus load in the liver was 1.4 log_{10} genomes/mg and in the spleen was 1.3 log_{10} genomes/mg. In contrast, in infected pups born to negative-control dams, the mean virus load in the liver was 3.8 log_{10} genomes/mg and in the spleen was 4.0 log_{10} genomes/mg (table 2).

**DISCUSSION**

Because of the ability of GPCMV to cross the placenta and cause infection and disease in utero, the guinea pig provides a uniquely useful model for study of CMV vaccine strategies. A number of studies have examined the protective efficacy of vaccines consisting of native GPCMV proteins purified by immunoaffinity column, lectin column chromatography, or detergent solubilization of virus and dense body fractions. All were administered with Freund’s adjuvant [24–26], an adjuvant not approved for human use. All of these strategies have proven useful in protecting against congenital GPCMV infection and/or disease, with the extent of protection dependent on the strain of guinea pig used, timing of viral challenge, and study end points examined. However, the general lack of detailed molecular characterization of the GPCMV genome has precluded subunit-vaccine studies using recombinant expression strategies. With the recent successful cloning and expression of the GPCMV homologs of the vaccine targets gB and GP83 [27–29], the present study was undertaken to evaluate the protective efficacy of these gene products against congenital CMV infection.

Previous studies using the murine CMV (MCMV) model have supported the concept that subunit vaccines using cloned, recombinant expression technologies are capable of conferring protection against CMV disease. A vaccinia recombinant expressing MCMV gB provided protection against lethal MCMV challenge in BALB/c mice when administered as a vaccine [34]. Studies have also examined protection against MCMV disease after vaccination with DNA vaccines against CTL targets; interestingly, in this model, homologs of immediate-early proteins and the MCMV UL84 homolog conferred the best protection, whereas the MCMV UL83 vaccine was generally ineffective [35–38]. Since MCMV does not cross the placenta or cause congenital infection, extrapolating these results to the prevention of congenital HCMV infection is problematic [39]. The present study has examined the value of maternal DNA vaccination against congenital CMV infection and disease. In liveborn pups, gB vaccine was found to provide protection against congenital infection, and, when congenital infection did occur, viral DNA load was lower in pups born to gB-vaccinated guinea pigs, compared with that in controls. These observations suggest that immune responses to gB play a key role in protective maternal immunity during pregnancy.

One observation of interest in gB-vaccinated dams was the important influence of the magnitude of the antibody response on GPCMV-induced pup mortality. Thus, there was no pup mortality (0/13) in pups born to gB-vaccinated dams with a high-titer ELISA antibody response (>3.4 log_{10}). In contrast, 50% overall pup mortality was noted in pups born to gB-vaccinated dams with ELISA titers =3.4 log_{10} (table 1). Although differences in the magnitude of the gB response correlated with outcome, all vaccinated dams made antibodies capable of immunoprecipitating the full gB complex, including the carboxy-terminal moiety, gp58, the region of the gB molecule that appears to encode neutralization-related epitopes [27]. The observation of the critical importance of antibody response in fetal protection is consistent with previous reports in the guinea pig model using passive antibody transfer studies and warrants additional study of alternative gB-expression strategies [31, 40]. Future studies of DNA vaccine designed to optimize the immunogenicity of the gB plasmid may therefore be warranted. In addition to the effect of vaccination on pup

<table>
<thead>
<tr>
<th>Group</th>
<th>Litters, no.</th>
<th>Liveborn pups, no.</th>
<th>GPCMV-infected pups, no. (%)</th>
<th>Liver Tissue virus load, mean ± SD, log_{10} genomes/mg of tissue</th>
<th>Spleen Tissue virus load, mean ± SD, log_{10} genomes/mg of tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>26</td>
<td>20 (77)</td>
<td>3.8 ± 1.9</td>
<td>4.0 ± 2.0</td>
</tr>
<tr>
<td>UL83 vaccine</td>
<td>8</td>
<td>25</td>
<td>17 (68)</td>
<td>1.4 ± 1.4</td>
<td>1.3 ± 0.9</td>
</tr>
<tr>
<td>gB vaccine</td>
<td>10</td>
<td>27</td>
<td>11 (41)</td>
<td>1.8 ± 1.4</td>
<td>1.3 ± 0.5</td>
</tr>
</tbody>
</table>

NOTE. Liveborn pups were killed within 72 h of delivery, and liver and spleen were evaluated for presence of GPCMV DNA by quantitative competitive polymerase chain reaction.

a P < .05, vs. control (Fisher’s exact test).
b P < .005, vs. control (Student’s t test).
c P < .01, vs. control (Student’s t test).
mortality in a subset of gB-vaccinated dams, these data clearly indicate an effect of gB vaccine on the congenital CMV infection rate in liveborn pups and on the magnitude of virus load in infected pups. These data are of particular relevance to HCMV-vaccine studies, because congenital infection, and not mortality or disease, is likely to be the end point that is most relevant for congenital HCMV infection, a disease that rarely causes mortality. The data indicating reduction of fetal virus load in congenitally infected pups is also relevant to HCMV disease, since evidence in HCMV-infected infants suggests that virus load may be predictive of neurodevelopmental sequelae [41].

In contrast to gB vaccine, DNA vaccine based on the GPCMV UL83 homolog, GP83, provided no significant protection against infection. Several possibilities could explain the lack of efficacy of the GP83 vaccine. The epidermal (gene gun) administration of DNA vaccine, although a more efficient route for induction of strong neutralizing-antibody responses [42], may be less useful for CTL targets, because of the Th-2 cytokine bias induced by this approach. Alternatively, the GP83 homolog, like the MCMV M83 homolog, may not represent the dominant CTL target in the setting of GPCMV infection. Since a DNA vaccine based on the MCMV UL84 homolog, M84, appears to be more strongly protective than M83 DNA vaccine [36], testing a DNA vaccine based on the GPCMV UL84 homolog may prove of interest. Detailed analyses of T cell responses to GPCMV infection may provide useful insights into logical future subunit-vaccine targets. Interestingly, however, although preconception vaccination with GP83 did not decrease either pup mortality or the rate of congenital infection, there were statistically significant reductions in virus load among liveborn, congenitally infected pups, compared with those in liveborn, infected pups born to negative-control dams (table 2). Thus, although GP83 vaccine did not reduce the incidence of vertical transmission, it did appear to modify the magnitude of viral transmission in newborn pups, suggesting the need for future evaluation of GP83-vaccine approaches in this model. Evaluation of alternative vaccine approaches, such as in administration of DNA vaccine, or vectored approaches, such as vaccinia virus, may be useful in clarifying the role that the UL83 homolog plays in protective immunity against congenital GPCMV infection.

Although the guinea pig provides a useful model for a variety of congenital infections—including CMV, toxoplasmosis, and syphilis [43–45]—there have been few evaluations of subunit-vaccine strategies using this small-animal model. In the guinea pig model of congenital toxoplasmosis, a subunit vaccine based on the SAG protein was found to provide protection against vertical transmission [45]. As noted, purified native GPCMV proteins [34–36] have shown efficacy as vaccines against congenital GPCMV infection and disease. The present study, however, represents the first report of the efficacy of a subunit vaccine using recombinant expression technology for congenital CMV infection. Furthermore, these data represent the first report of the efficacy of any DNA vaccine for a congenital infection. In addition to confirming the potential value of gB subunit vaccines for prevention of congenital CMV infection, these data provide support for the continued development of CMV DNA vaccines for human use [46, 47]. Continued investigation of subunit vaccines in the GPCMV model should help to prioritize which strategies are likely to be most useful in development of HCMV vaccine.

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References

In an article in the 15 December 2003 issue of the *Journal* (Schleiss MR, Bourne N, Bernstein DL. Preconception Vaccination with a Glycoprotein B (gB) DNA Vaccine Protects against Cytomegalovirus (CMV) Transmission in the Guinea Pig Model of Congenital CMV Infection. J Infect Dis 2003; 188:1868–1874), the “Data” footnote to table 1 (p. 1871) should read as follows: “Data are no. of guinea pigs [not mice], unless otherwise noted.” The publisher regrets this error.