Characterization of *in vitro* and *in vivo* Antiviral Activity of Lactoferrin and Ribavirin upon Hantavirus

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ABSTRACT. Mechanisms of anti-hantaviral activities of bovine lactoferrin (LF) and ribavirin (Rbv) were investigated. Hantavirus focus formation at 48 hr was 15% of the control in cells treated with 400 $\mu\text{g}/\text{ml}$ LF for 1 hr at 37°C prior to viral infection. Post infection treatment with 100 $\mu\text{g}/\text{ml}$ Rbv also inhibited the focus formation to 2.5% of the control. Combined LF pre- and Rbv post-infection treatment completely inhibited focus formation. Viral glycoprotein (G2) and nucleocapsid protein (NP) syntheses were delayed in LF pre-treated cells up to 24 hr post infection (hpi) but became comparable to the control by 48 hpi. Further, LF inhibited viral shedding at 24 hpi but did not inhibit shedding after 48 hpi. However, Rbv was able to inhibit synthesis of viral proteins, (+) and (–) strand RNAs also inhibited viral shedding after 24 hr. These results suggest that LF inhibits viral adsorption to cells, while Rbv inhibits viral RNA synthesis. For *in vivo* trials of LF and Rbv, LF pre- and Rbv post-treatment were evaluated in suckling mice infected with hantavirus, of which 7% survived. LF concentrations of 40 and 160 mg/kg administered prior to viral challenge improved survival rates to 15% and 70%, respectively for single administration and 85% and 94%, respectively, for double administration. Rbv concentrations of 25 and 50 mg/kg gave survival rates of 68% and 81%, respectively. This suggests that both LF and Rbv are efficacious in hantavirus infection *in vivo*.

KEY WORDS: confocal microscopy, hantavirus, lactoferrin, ribavirin, zoonosis.

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Hantavirus is a zoonotic, rodent-borne agent, which causes severe human diseases. Infected rodents are asymptomatic and can harbor the virus for a long period, shedding the virus in their excreta. To date, two distinct manifestations of hantavirus infection are known: one is hemorrhagic fever with renal syndrome (HFRS) endemic to Eurasia and the other is hantavirus pulmonary syndrome (HPS) endemic to the Americas [4, 5, 18, 19, 26, 29, 33].

Hantavirus is an enveloped negative-sense RNA virus belonging to the family *Bunyaviridae* [15, 34]. The genome consists of tripartite, single stranded RNAs which are designated S, M, and L RNA which encode nucleocapsid protein, glycoproteins (G1 and G2), and RNA polymerase, respectively [8, 32]. Such a characteristic as RNA synthesis is thought to be consistent in the family *Bunyaviridae*, to which the hantavirus belongs. One common characteristic of this family is RNA replication occurring in the cytoplasm. After the genome sense RNA is uncoated in the cytoplasm, the plus-strand RNA [mRNA and complementary RNA (cRNA)] is synthesized. Viral proteins are translated from mRNA, and genome sense RNA is replicated by using cRNA as the template. Lactoferrin (LF), an antibacterial, iron-binding glycoprotein, was reported to manifest a broad antiviral activity [2, 23, 44]. In this study, we found that LF also inhibits *in vitro* hantavirus infection [28]. The mechanism appears to involve inhibition of virus adsorption to cells.

Ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a synthetic guanosine analog having broad spectrum antiviral properties to DNA and RNA viruses, acts through inhibition of viral RNA synthesis [10, 31, 39, 40]. We also found that Rbv exhibits *in vitro* anti-hantaviral activity [13, 28].

To further investigate possible mechanisms of anti-hantaviral activities of LF and Rbv, these reagents were assessed *in vitro* in greater detail. Furthermore, these reagents were tested *in vivo* in mouse model. We found that mice treated with LF prior to infection or with Rbv at the time of infection, demonstrated increased survival rates. This is the first report describing *in vivo* inhibition of hantavirus by LF.

MATERIALS AND METHODS

Cells, viruses, and antiviral agents: Vero E6 cells were cultured in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 5% fetal calf serum (ICN Biomedical Inc., Aurora, USA) and 0.03% L-glutamine (MEM) at 37°C. SR-11 strain Seoul type hantavirus was propagated in Vero E6 cells and culture media of the infected cells stored at –80°C as virus stock.

The virus foci were formed under MEM containing 1.5% carboxymethyl cellulose (CMC/MEM; Wako Pure Chemicals Ltd., Osaka, Japan) for 5 days at 37°C. In our previously published report we described in detail the visualization of foci [28].

Bovine lactoferrin (LF; Wako Pure Chemical Industries,

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Ltd., Osaka, Japan) and ribavirin (Rbv; Yamasa Corp., Choushi, Japan) were evaluated in SR-11 infected cells.

Viral focus formation: Monolayers of Vero E6 cells were established on 24 well slides which were pretreated with 400 $\mu\text{g/ml}$ LF and/or 100 $\mu\text{g/ml}$ Rbv following infection with 5 ml SR-11 for various periods of time. The time periods were as follows; untreated control, LF pretreatment one hr prior to virus infection (–1 to 0 hr LF), Rbv administered after viral infection for up to 48 hr (0 to 48 hr Rbv), and the combination of the above LF and Rbv treatments (–1 to 0 hr LF with 0–48 hr Rbv). In the case of LF pretreatment, at 0 hr, the LF was replaced with LF free 1.5% CMC-MEM. Over the course of the 48 hr experiment, cells were fixed with equal parts of acetone and methanol for 20 min at 0, 1, 2, 12, 24 and 48 hr. The 24 well slides were air-dried and stored at -40°C until use. The slides were reacted with E5/G6 monoclonal antibody [1] for 1 hr at 37°C , washed with PBS (pH 7.6) and treated with anti-mouse IgG FITC conjugate. Foci were counted under a fluorescence microscope.

Evaluation of viral yield in cell culture fluid: Treatments of LF, Rbv, and LF/Rbv in combination were evaluated against Seoul virus infection *in vitro*. Vero E6 cell monolayers grown in 25 cm^2 flasks were pretreated for 1 hr with 400 $\mu\text{g/ml}$ LF. SR-11 was inoculated on the cell monolayers (moi 0.001) and the cells were incubated in MEM for 1 hr at 37°C under 5% CO_2 . The inoculum was discarded and the infected cells were cultured. The culture fluids were collected at 0, 1, 2, 12, 24, and 48 hpi and stored at -80°C until use. The collected culture fluids were serially diluted and inoculated onto Vero E6 monolayers grown in 96 well plates. The cells were cultured in CMC-MEM for 5 days at 37°C under 5% CO_2 and fixed with acetone:methanol (1:1). The virus foci were visualized with anti-hantavirus nucleocapsid protein monoclonal antibody (NP); E5/G6 [1] and anti-mouse IgG FITC conjugate. The foci were counted under a fluorescence microscope. The virus yields were determined by calculating the means of four wells.

For Rbv, the time of treatment was subsequent to viral infection. Following infection of SR-11 to Vero E6 cells, 100 $\mu\text{g/ml}$ Rbv was added and cultured for 48 hr. The calculation of virus yields were determined by the above mentioned method.

For the combined treatment of LF/Rbv, the order of treatment and infection was as follows; LF pretreatment, SR-11 adsorption, and Rbv post-treatment.

Northern blot analysis: The 3' end of the Seoul virus S genome (1–805 nt) was cloned into pCR II (Invitrogen, Carlsbad, CA). The digoxigenin (DIG) labeled (–) and (+) RNA probes were prepared by DIG RNA Labeling Kit (Roche, Alameda, U.S.A.).

In vitro Rbv efficacy over time was evaluated *in vitro* on monolayers of Vero E6 cells grown in 25 cm^2 flasks. Vero E6 cells infected with SR-11 were incubated with the antiviral reagent for various lengths of time. The time periods were as follows; untreated control, –1 to 1 days post infection (dpi), 0 to 1 dpi, 0 to 2 dpi, 0 to 3 dpi, 0 to 4 dpi, and 0 to 5 dpi. At the indicated dpi, Rbv MEM was replaced with

Rbv free MEM. At 5 dpi, the cells were washed 3 times with PBS, trypsinized with a 0.3% solution in PBS, collected with 10 ml MEM, and centrifuged to obtain a cell pellet. Total RNA was obtained by use of Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's recommendations, and stored at -80°C . Total RNA (5 μg) was denatured with a solution containing formamide, formaldehyde, 10×3 -[N-morpholino] propanesulfonic acid (MOPS), ethidium bromide (EtBr) and double distilled water (DDW) for 5 min at 65°C . Samples were then placed on ice for 5–10 min and were electrophoresed in 1% agarose gels. Gels were observed under UV and washed with $10 \times \text{SSC}$ for 30 min. RNA was blotted onto a nylon membrane (Hybond-N+, Amersham, UK) overnight, exposed to UV for 3 min, baked at 80°C for two hrs, and prehybridized with 10 ml hybridization solution for 1 hr at 68°C . During hybridization, one membrane was treated with 2 ml (–) RNA probe for detecting plus strand viral RNA, the other with 2 ml (+) RNA probe for detecting minus strand RNA in 10 ml hybrid solution, then incubated 12–16 hr at 68°C . Both membranes were washed vigorously and sealed in a hybridization bag and rocked for 30 min. The membranes were first prewashed, then washed 3 times for 15 min. The membranes were incubated with chemiluminescent substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (CSPD; TROPIX, MA, U.S.A.) for 5 min at room temperature, sealed in hybridization bags, incubated for 15 min at 37°C , and then exposed onto X-ray film.

Confocal imagery: Vero E6 cell monolayers were grown in 24-well (Eirre Sci. Co., NH) or 8-chamber slides (Becton Dickinson, NJ) in MEM. The cell monolayers were inoculated with 5 ml SR-11 per well for 6 hr at 4°C and the inoculum was removed. The groups of cells were; untreated controls, pretreated with 400 $\mu\text{g/ml}$ LF for 1 hr, post-treated with 100 $\mu\text{g/ml}$ Rbv for 1–48 hpi, and that receiving the combined treatment of LF and Rbv. The infected Vero E6 cells were incubated for 0, 1, 2, 12, 24, or 48 hr after adsorption at 37°C . The monolayers were rinsed with phosphate buffered saline (PBS; pH 7.6) and fixed for 20 min in a mixture of equal parts of acetone and methanol. The slides were air-dried and stored at -40°C until used. The cells were examined by confocal imagery.

In brief, concerning the confocal imagery procedure, the fixed cells were reacted with mouse monoclonal antibodies, 1G8 to hantavirus G2 proteins [46], E5/G6 to NP [1] or normal mouse serum for 1 hr at 37°C , washed in PBS, and followed by FITC conjugated anti-mouse serum IgG goat antibody (ICN Pharmaceuticals Inc., Aurora, USA) for 1 hr at 37°C . The antiviral effects of lactoferrin (LF; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and ribavirin (Rbv; courtesy of Yamasa Corp., Choushi, Japan) were observed individually or concomitantly by microradiance confocal laser imagery (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and localization of GP and NP translation was examined.

Evaluation of LF and Rbv in mice infected with SR-11:

Fig. 1. Inhibition of hantavirus focus formation by LF and Rbv. Cells grown in 24-well slides were treated with 400 $\mu\text{g}/\text{ml}$ LF and/or 100 $\mu\text{g}/\text{ml}$ Rbv following infection with Seoul virus. The treatment regimens were as follows: MEM control ($\square$); -1 to 0 hr LF ($\circ$); 0 to 48 hpi Rbv ($\bullet$); -1 to 0 hr LF and 0 to 48 hpi Rbv (Δ). Each symbol shows mean $\pm$ s.e (although often not visible; $n=8$). Cells were fixed at 0, 1, 2, 12, 24 and 48 hr.

Suckling ICR mice were subcutaneously (sc) administered 10, 40, or 160 mg/kg LF at 1 and/or 2 day(s) old (d.o.) and sc inoculated with SR-11 (100 FFU) at 3 d.o. Mice were observed for 25 days following infection. For Rbv evaluation, 3 d.o. mice were administered 25 or 50 mg/kg Rbv 1 hr after SR-11 infection and observed as above. All animal experiments were performed according to the experimental animal guidelines of the Graduate School of Veterinary Medicine, Hokkaido University.

RESULTS

Inhibition of hantavirus focus formation by LF and Rbv:

Hantaviral focus formation was evaluated in cells pretreated with 400 $\mu\text{g}/\text{ml}$ LF for 1 hr at 37°C. Focus numbers were 15% of those seen in the control group at 48 hr (Fig. 1). From 24 hpi, cells pretreated with LF exhibited increasing foci. LF inhibited an early stage of infection such as adsorption as indicated in our previous study [28] but could not inhibit focus formation once the virus was adsorbed to the cells. However, Rbv could inhibit focus formation even though the virus was allowed to attach to the cell. Cells treated with 100 $\mu\text{g}/\text{ml}$ Rbv maintained hantavirus at low levels from an initial 11 foci at 2 hpi to 5 foci/well at 48 hpi. No foci were observed in cells treated with the combination of LF/Rbv after 12 hpi (Fig. 1).

Inhibition of hantavirus RNA synthesis by Rbv: In order to

A

B

Fig. 2. Northern blot analysis for hantaviral RNA synthesis. Vero E6 cells grown in 25-cm² flasks were infected with a uniform concentration of SR-11 and cells were treated with 100 $\mu\text{g}/\text{ml}$ Rbv at different times of onset column: 1) control, 2) -1 to 1 dpi, 3) 0 to 1 dpi, 4) 0 to 2 dpi, 5) 0 to 3 dpi, 6) 0 to 4 dpi, 7) 0 to 5 dpi. Cell pellets were obtained at 5 dpi and total RNA extracted. Both the positive (A) and negative (B) strand genomes syntheses were equally prohibited.

Fig. 3. Detection of G2 protein in cells infected with hantavirus, Vero E6 monolayers were infected with SR-11 strain and treated with: MEM, LF, Rbv, or LF/Rbv. Cells were fixed at 2, 12, 24 and 48 hr after infection. Infected cells were stained with G2 mouse monoclonal antibody (1G8) followed by FITC-conjugated anti-mouse immunoglobulin and observed under a confocal microscope.

Fig. 4. Detection of NP in cells infected with hantavirus. Under the same conditions as Fig. 3, cells were incubated with anti-NP monoclonal antibody (E5/G6) followed by FITC-conjugated anti-mouse immunoglobulin and observed under a confocal microscope.

Fig. 5. Virus yield in culture fluid treated with LF and/or Rbv. Culture fluid was sampled at 1, 12, 24, 48, 72, 96, and 120 hpi. Vero E6 monolayers were treated with 400 $\mu\text{g}/\text{ml}$ LF and/or 100 $\mu\text{g}/\text{ml}$ Rbv and viral yield determined by titration. The reagents were as follows: control (Δ); -1 to 0 hr LF ($\square$); 0 to 5 dpi Rbv ($\bullet$); -1 to 0 hr LF and 0 to 5 dpi Rbv ($\circ$). Each symbol shows mean $\pm$ s.e (n=8).

Fig. 6. Survival curve of hantavirus infected mice with Rbv administration. Suckling ICR mice infected with SR-11 strain (100 FFU/mouse) were subcutaneously (SC) administered in MEM ($\square$); 25 mg/kg Rbv (Δ); or 50 mg/kg Rbv ($\circ$) at 3 days old. Rbv treatment was initiated 1 hr after infection.

determine whether Rbv acts on viral RNA synthesis, Northern blot analysis was performed on RNAs extracted from SR-11 infected Vero E6 cells at 5 dpi. DIG-labeled strand-

Fig. 7. Survival curve of hantavirus infected mice with LF single administration. Suckling ICR mice were administered (SC) with 0.1 ml of the indicated concentrations of MEM ($\circ$); or 160 ($\square$), or 40 (Δ) mg/kg LF at 2 days old. All mice were inoculated with 0.1 ml SR-11 containing 100 FFU on day 3.

Fig. 8. Survival curve of hantavirus infected mice with LF double administration. Suckling ICR mice were treated with various concentrations of LF at 1 and 2 days old. Double administration of the MEM control ($\circ$); or 160 ($\square$), or 40 (Δ), or 10 mg/kg LF ($\bullet$) were administered SC 1 and 2 days prior to virus challenge at 3 days old.

specific RNA probes were utilized to detect genomic and antigenomic viral RNA (Fig. 2). Hantaviral genomic and antigenomic RNA syntheses were equally inhibited by Rbv

treatment. Pretreatment and early treatment (0–1 and 0–2 dpi) with Rbv exhibited no effect on viral RNA synthesis. However, 0–3, 0–4 and 0–5 dpi treatments inhibited viral RNA synthesis (Fig. 2, lanes 5 to 7).

Inhibition of hantavirus protein expression by LF and Rbv: Viral glycoprotein (G2) expression was observed under a confocal laser microscope. At 2 hpi, G2 was detected in all cells treated with MEM, LF, Rbv, and LF/Rbv (Fig. 3). The control and LF pretreated cells similarly showed increasing G2 expression by 48 hpi. Viral G2 expression in Rbv treated cells was inhibited after 12 hpi. The protein expression in cells treated with the combination of LF/Rbv was completely inhibited from 12 hr on.

Similar results were obtained concerning expression of NP as seen in Fig. 4.

Inhibition of virus shedding by LF and Rbv: Viral yield of culture fluid at 24 hpi revealed a 10 fold difference between the control and LF pre-treated cell culture fluid (Fig. 5). This initial difference decreased over a period of time and from 48 hpi, these levels equalled those of the untreated cells. Culture fluid obtained from cells treated with Rbv demonstrated inhibition of viral shedding from 24 hr. Combined LF/Rbv treatment revealed synergistically reduced virus yield by 120 hpi.

Efficacy of Rbv in mouse model: In mice infected with Seoul virus, the anti-hantaviral efficacy of ribavirin was evaluated by administration at 1 hpi. The survival rates of mice sc infected with 100 focus forming units (FFU) (30 LD₅₀) of SR-11 were 6.7% for a period of 25 days (Fig. 6). The survival rates in Rbv treated mice were dose dependent. The administration of 50 and 25 mg/kg Rbv gave survival rates of 81.8 and 68.8%, respectively.

Efficacy of LF single and double administration: Under the conditions similar to those of Rbv administration, suckling mice were sc administered a single dose of 160 or 40 mg/kg LF or MEM 1 day prior to viral challenge (Fig. 7). Survival rates of these groups were 70, 15.4 and 6.7%, respectively. Suckling mice were sc double administered 160, 40, or 10 mg/kg LF or MEM 2 days and 1 day prior to viral challenge (Fig. 8). The survival rates of these groups were 94.1, 85.7, 50 and 6.7%, respectively, in a dose dependent manner.

DISCUSSION

In this study, antiviral agents were tested against SR-11 strain of hantavirus Seoul type. The reagents used were bovine lactoferrin, an iron binding glycoprotein found to have anti-bacterial, -fungal and -viral properties [2, 23, 44] and ribavirin, a synthetic guanosine analog noted for its broad spectrum antiviral properties to both DNA and RNA viruses through inhibition of viral RNA synthesis [10, 31, 39, 40]. Rbv is known to inhibit viruses such as arenavirus, coxsackievirus, Influenza viruses, La Crosse virus, and respiratory syncytial virus and other viruses [3, 12–14, 24, 27, 30, 39, 40, 42].

We previously reported that LF and Rbv inhibited han-

Table 1. Summarization of *in vitro* antihantaviral characteristics of LF and Rbv

Activity inhibited	LF	Rbv
Adsorption	+	–
Focus formation	±	+
RNA synthesis	–	+
Protein synthesis	±	+

tavirus infection *in vitro* [28]. The concentration of LF was previously found to have no cytotoxicity even at 5 mg/ml [28] and Rbv demonstrated minimal cytotoxicity when the dosage was under 2.5 mg/ml as determined by trypan blue exclusion test (data not shown). Therefore, the amounts used in the present experiment were well below the cytotoxic levels. We further confirmed that inhibition of hantavirus infection by LF was due to reduction of virus adsorption as found in polio virus, herpes simplex type 1, human cytomegalovirus, and HIV [9, 11, 21, 22, 43] as opposed to Hepatitis C in which LF has been found to directly bind viruses and inhibit viral infection [16, 17, 45].

To further elucidate the efficacy and possible mechanisms of LF and Rbv upon hantavirus, these reagents were evaluated *in vitro* and *in vivo* in greater detail. *In vitro*, we examined the effects of both reagents upon focus formation, RNA synthesis, antigen production and infectious titer of hantavirus.

LF or Rbv treatment could reduce the number of foci but could not completely inhibit their formation. While no foci were observed in the combined treatment of LF/Rbv from 12–48 hr, thus indicating a synergistic effect of these reagents. This corroborates our previous findings that pretreatment with LF and subsequent treatment with Rbv synergistically reduces viral focus formation in early infections [28].

The genome minus strand RNA and plus strand RNA syntheses were greatly and equally reduced by Rbv, but viral RNA synthesis was not completely inhibited (Fig. 2). However, in this condition, viral shedding was completely prohibited [28]. While in the control, viral shedding was continuous through 5 dpi (Fig. 5). Other investigators have reported subsequent elevations of viral RNA to original levels following removal or deterioration of Rbv [27, 35]. This indicates Rbv inhibits viral transcription and reduces infectivity, however, Rbv solely, cannot completely eliminate the virus.

Hantavirus G2 and NP protein translation was not reduced by LF once the infection was established in each cell (Figs. 3 and 4). However, Rbv demonstrated a pattern in which the foci of infected cells became more discreet over time, few foci were observed by 48 hr. This supports the hypothesis that Rbv actively inhibits viral protein expression within the cell and does not inhibit viral adsorption [14, 40]. The absence of viral protein expression by Rbv does not exclude the presence of viral RNA. Under the conditions of reduced viral RNA synthesis, the virus may not express sufficient amounts of protein to be detected by IFA.

The combination of LF/Rbv revealed no discernible protein expression from 12 hr, for G2 and NP, clearly emphasizing the synergistic effects of the two reagents at the level of protein synthesis.

Viral yield was determined in SR-11 infected Vero E6 cells. The virus yield of culture fluid again demonstrated an early hantaviral inhibition by LF at 24 hr which becomes less effective over time, equalling the control by 48 hr (Fig. 5). This further supports the concept that LF inhibits adsorption although, once a viral infection is established, the LF is no longer efficacious *in vitro*. However, Rbv which has been demonstrated to act within the cell against several viruses [14, 40], shows a greatly reduced yield continuously from 24–120 hr. The combination of LF/Rbv also reveals a reduced viral yield which slightly indicates synergy by 120 hr (Fig. 5).

Mice administered Rbv 1 hpi revealed an improved survival rate. The administration of 50 and 25 mg/kg Rbv gave 81.8 and 68.8% survival rates, respectively (Fig. 6). The same result was reported by Huggins *et al.* [13]. This may be due to the inhibition of the viral RNA synthesis, which we observed in the *in vitro* evaluation portion of this study.

Mice administered LF one day prior to hantavirus inoculation had an improved survival rate of 70% for 160 mg/kg (Fig. 7). As seen in *in vitro*, this may be due in part to the inhibition of viral adsorption to target cells *in vivo* as well. Double administration of LF enhanced the survival rate. The 160 and 40 mg/kg double administrations resulted in 94.1% and 85.7% survival rates, respectively (Fig. 8). The difference between single and double administrations of LF may correlate to insufficient time for complete LF absorption in the single dose mice or an accumulative effect of LF in the body from the two administrations. Another possible difference may be that a certain period of time is necessary for LF to activate the immune system. LF has been demonstrated to enhance cytotoxic activities of monocytes [2, 25] and NK cells [6, 7, 36, 37]. To evaluate augmentation of the immune system by LF, another *in vivo* experiment should be carried out by using older but susceptible mice, or by quantitative virus detection assay.

This study presents findings of anti-hantaviral activity by Rbv and LF. Further studies concerning LF activity may provide insight into effective clinical uses as a protective and a possible curative agent of hantavirus. LF and Rbv combination therapy may even be used as a measure for hantavirus infection in humans.

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