Expression of interleukin-8 receptors in endometriosis

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BACKGROUND: Although the etiology of endometriosis is not well understood, chemokines and their receptors are believed to play a role in its pathogenesis. Therefore, we aimed to investigate the expression and localization of interleukin-8 (IL-8) receptors CXCR1 and CXCR2 in eutopic and ectopic endometrial tissues of women with endometriosis, and in endometrium of women without endometriosis. METHODS: Ectopic (n = 27) and homologous eutopic endometrium (n = 25) from women with endometriosis and endometrium from women without endometriosis (n = 27) were used for immunohistochemical analysis of CXCR1 and CXCR2. RESULTS: In normal endometrium, epithelial CXCR1 and CXCR2 immunostaining intensities were similar in the proliferative and secretory phase. Stromal CXCR1 expression was less than epithelial expression and did not show cyclical difference. No stromal CXCR2 expression was observed. In eutopic endometrium of women with endometriosis compared to endometrium of women without endometriosis, there was a significant increase in both proliferative and secretory phases for epithelial CXCR2 expression, and in proliferative phase for CXCR1 expression (P < 0.05). Both receptor immunoreactivities were significantly increased in the epithelial cells of ectopic endometrial tissues compared to that of normal endometrium (P < 0.05). CONCLUSIONS: These findings suggest that IL-8 and its receptors may be involved in the pathogenesis of endometriosis.

Key words: endometriosis/endometrium/immunohistochemistry/interleukin-8 receptors

Introduction

Endometriosis is defined as the presence of both endometrial stromal and glandular cells outside the uterine cavity, mainly in the pelvis. It is estimated that this disorder affects at least 10–20% of all women of reproductive age; however, its incidence may be as high as 40–50% in infertile women (Oral and Arici, 1996). The pathogenesis of endometriosis still remains controversial despite extensive research. However, immune factors as well as hormonal, genetic and environmental factors seem to be involved in the pathogenesis of endometriosis (Kennedy et al., 1995; Noble et al., 1996; Mayani et al., 1997; Hill et al., 1988).

Chemokines, a subclass of the cytokine superfamily, are small polypeptides produced by several cell types including immune cells, mesothelial cells, endometrial stromal and glandular cells, and trophoblasts (Kayisli et al., 2002). Chemotaxis is one of the hallmarksof chemokines. Additionally angiogenesis, cellular proliferation, differentiation and apoptosis are other known functions (Arici et al., 1998a; Koch, 1998; Kayisli et al., 2002; Selam et al., 2002). Chemokines are likely to be involved in the regulation of normal reproductive processes such as ovulation, menstruation, endometrial remodelling and implantation (Runesson et al., 1996; Garcia Velasco and Arici, 1999a). It is believed that they also play important roles in pathological processes such as endometriosis (Garcia Velasco and Arici, 1999a).

Chemokines bind to their membrane receptors to exert their effect, and the levels of the receptors modulate part of their actions. Although the intracellular signalling pathways of chemokine receptors are still poorly understood, it has been postulated that the structure and function of chemokine receptors are similar to guanine nucleotide binding proteins (G-proteins) (Premack and Schall, 1996). These receptors contain seven hydrophobic segments that span the plasma membrane, a characteristic of G proteins (Holmes et al., 1991; Gao et al., 1993).

Interleukin (IL)-8, an α-chemokine with neutrophil chemotactic/activating and T cell chemotactic activity both in vivo and in vitro, is believed to play a role in both endometrial physiology and endometriosis (Arici et al., 1998a; Iwabe et al., 1998). IL-8 acts as an autocrine growth factor in the endometrium and may also play a role in the pathogenesis of endometriosis by promoting the vicious cycle of endometrial...
cell attachment, invasion, cell growth, immune protection and further secretion of this cytokine in ectopic sites (Arici et al., 1998b; Garcia Velasco and Arici, 1999b,c; Selam et al., 2002).

CXCR1 and CXCR2 are the receptors for IL-8. In neutrophils, these two receptors mediate different functional responses to IL-8. Both receptors are involved in affecting intracellular calcium [Ca^{2+}] concentration, releasing of enzyme granules, and chemotaxis in response to IL-8. On the other hand, O_2^- release and the activation of phospholipase D in response to IL-8 depend particularly on CXCR1 (Chunharapai et al., 1994; Hammond et al., 1995; Jones et al., 1996; Green et al., 1996).

Just as coordination of chemokine-chemokine receptor interactions play important roles in both physiologic and pathologic processes, it is plausible to speculate that these interactions may contribute to the pathogenesis of endometriosis. Therefore, in the present study, we aimed to investigate the expression and localization of IL-8 receptors CXCR1 and CXCR2 in eutopic and ectopic endometrial tissue of women with endometriosis and endometrial tissue of women without endometriosis, and to compare their expression among these groups.

Materials and methods

Collection of tissue

Endometriosis samples were collected from 27 women (mean age, 43.1; range, 25–53) during laparoscopy or laparotomy. Distribution of endometriosis tissues were as follows: ovarian endometriomas (n = 15), peritoneal implants (n = 6), fallopian tube implants (n = 3) and uterine serosal implants (n = 3). From 25 out of 27 women with endometriosis, eutopic endometrium samples were also obtained by endometrial biopsy or hysterectomy. As a control group, endometrial tissues were obtained from 27 fertile women (mean age, 42.7; range, 31–53) with normal menstrual cycles undergoing laparoscopy or hysterectomy for benign gynecological conditions other than endometrial disease. Indications for surgery for the control group were as follows: leiomyomata (n = 16), voluntary sterilization (n = 8) myometrial hypertrophy (n = 2) and benign adnexial mass (n = 1). These women had no visible pelvic inflammation or endometriosis at laparoscopy or laparotomy. The day of the menstrual cycle was established from the women’s menstrual history and was confirmed by endometrial histology using the criteria of Noyes et al. (1950). The presence of endometriosis was confirmed by histopathological examination of the implants. All endometrial samples were grouped according to the menstrual cycle phase: proliferative (days 1–14 of the cycle) and secretory (days 15–28). Among eutopic endometrium of women with endometriosis, 12 were in the proliferative and 13 were in the secretory phase, whereas in the control group, 9 were in the proliferative and 18 were in the secretory phase of the cycle.

Written informed consent was obtained from each woman before surgery using consent forms and protocols approved by the Human Investigation Committee of Yale University.

Immunohistochemistry

Formalin fixed slides were embedded in paraffin and cut into 5 μm sections. After being deparaffinized in xylene and rehydrated in a graded series of ethanol, slides were boiled in citrate buffer (10 mM, pH 6.0) for 30 min for antigen retrieval. Then, sections were immersed in 1% hydrogen peroxide in phosphate-buffered saline (PBS) for 15 min to block endogenous peroxidase. Slides were then incubated in a humidified chamber with blocking horse serum (LabVision, Fremont, CA) for 10 min at room temperature. Excess serum was drained and primary antibodies (murine monoclonal anti-human CXCR1 antibody immunoglobulin IgG2B, clone 5A12; Pharmingen, San Diego, CA), 500 μg/ml, 1:200 dilution in 1% bovine serum albumin in PBS (PBS–BSA); murine monoclonal anti-human CXCR2 antibody IgG2A clone 8311.211 (R&D Systems, Minneapolis, MN), 500 μg/ml, 1:200 dilution in PBS–BSA were added to the sections. For negative controls, normal mouse IgG isotypes were used at the same concentrations. Neutrophils were used as positive control for both CXCR1 and CXCR2. Sections were incubated for 1 h at room temperature in a humidified chamber. The sections were rinsed and washed three times for 5 min with PBS, and then biotinylated horse anti-mouse antibody (1.5 mg/ml; Vector Laboratories, Burlingame, CA) was added at 1:250 dilution for 45 min at room temperature. The antigen-antibody complex was detected by using an avidin–biotin–peroxidase kit (LabVision). Diaminobenzidine (3,3-diaminobenzidine tetrahydrochloride dihydrate; LabVision) was used as the chromogen and sections were counterstained with hematoxylin and mounted with Permount (Fisher Chemicals, Springfield, NJ) on glass slides.

Immunohistochemical staining for CXCR1 and CXCR2 was evaluated in a semiquantitative fashion [i.e. 0 (absent) to 3 (most intense)]. Epithelial and stromal cells were separately evaluated and scored. Vascular cells were individually evaluated. For each slide, an HSCORE value was derived by summing the percentages of stained cells at each intensity, multiplied by the weighted intensity of the staining, that is HSCORE = Σ Pi·i, where i is the intensity score and Pi is the corresponding percentages of the cells. In each slide, 10 different areas were evaluated under a microscope (50X magnification), and the percentage of the cells for each intensity within these areas was determined by two investigators at different times in a blinded fashion. The average score of the two was used.

Statistical analysis

Epithelial and stromal CXCR1 HSCORE values were normally distributed as tested by Kolmogorov–Smirnoff test. Differences between proliferative and secretory phase samples were analyzed using t-test. Differences in epithelial CXCR1 HSCORE values between normal endometrium, eutopic endometrium and endometriosis samples were analyzed using one-way ANOVA test and post hoc Bonferroni test for pairwise multiple comparisons.

Epithelial and stromal CXCR2 HSCORE values were not normally distributed as tested by Kolmogorov–Smirnoff test. Differences between proliferative and secretory phase samples were analyzed using Mann–Whitney Rank Sum Test and pairwise multiple comparisons were analyzed using nonparametric ANOVA on ranks (Kruskal–Wallis test).

All statistical analyses were performed using Sigmastat for Windows, version 3.0 (Jandel Scientific Corporation, San Rafael, CA). Data are presented as the mean ± SEM. Differences were considered to be significant at P < 0.05.

Results

CXCR1 and CXCR2 expression in normal endometrium

In normal endometrial samples, epithelial and stromal CXCR1 expression was observed in all of the nine proliferative samples, whereas 1 out of 18 secretory phase

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endometrial samples showed neither epithelial nor stromal staining. The stromal component of the endometrium stained with less intensity compared to the epithelial cells in both proliferative and secretory phase samples. CXCR1 immunoreactivity was diffuse in most samples and localized in the surface epithelium and glands throughout the menstrual cycle (Figure 1A and B). The epithelial staining was mostly cytoplasmic. The stromal staining was membranous and cytoplasmic, and was most intense particularly in the periglandular areas. There was no significant difference in epithelial and stromal CXCR1 HSCORE values between proliferative and secretory phases of the menstrual cycle. Myometrial compartment, smooth muscle cells of arterioles, venules and capillaries showed weak staining in most samples, contrasting with the CXCR1 staining.

On the other hand, four samples from the proliferative and four samples from the secretory phase showed no epithelial staining with CXCR2 antibody in normal endometrium. Moreover, none of the samples showed stromal immunoreactivity with this antibody. Contrary to the epithelial CXCR1 staining, CXCR2 staining was mostly focal, but staining characteristics were similar (Figure 2A and B). There were no differences in epithelial CXCR2 HSCORE values between proliferative and secretory phases of the menstrual cycle. Myometrial compartment, smooth muscle cells of arterioles, venules and capillaries showed weak staining in most samples, contrasting with the CXCR1 staining.

**CXCR1 and CXCR2 expression in eutopic and ectopic endometrium of women with endometriosis**

Epithelial and stromal CXCR1 immunoreactivity was observed in both proliferative and secretory phases of all eutopic endometrial samples of women with endometriosis. Similar to the normal endometrial samples, epithelial cells of the eutopic endometrium stained with higher intensity compared to the stromal cells. Although staining intensity was higher, staining characteristics were similar to normal endo-

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**Figure 1.** Representative micrographs of immunohistochemistry staining for CXCR1 in the eutopic and ectopic endometrium of women with and without endometriosis. In both eutopic and ectopic samples, epithelial staining intensity was more prominent than stromal staining. Diffuse, but weak cytoplasmic epithelial immunoreactivity was noted in the surface epithelium and glands throughout the menstrual cycle in normal endometrium samples. Stromal staining was mostly localized in periglandular areas (A, proliferative phase; B, secretory phase). In the eutopic endometrium of women with endometriosis staining intensity was higher throughout the menstrual cycle compared to the normal endometrium (C, proliferative phase; D, secretory phase). Highest staining intensity in both epithelial and stromal components was observed in ectopic endometrial implants (E). Scale bars represent 150 μm (A and D); 100 μm (B and C); 200 μm (E).
metrial samples; the epithelial staining was cytoplasmic, whereas the stromal staining was membranous and cytoplasmic, and was mostly detected around periglandular areas (Figure 1C and D). HSCORE value of the epithelial CXCR1 immunostaining was significantly higher in the proliferative phase of the eutopic endometrial samples of women with endometriosis compared to the proliferative phase normal endometrial samples (*P*, 0.05). In the secretory phase samples, there was a trend for higher epithelial HSCORE values in eutopic endometrium of women with endometriosis compared to normal endometrium (*P* = 0.10) (Figure 3). Stromal immunostaining with CXCR1 was also higher in the endometrium of women with endometriosis compared to normal endometrium in both menstrual cycle phases. Myometrium and vascular structures were also stained strongly with CXCR1, but no difference was observed in these structures between women with or without endometriosis.

All ectopic endometrial tissues were stained strongly for CXCR1. Similar to the autologous eutopic endometrium and normal endometrium, epithelial areas expressed higher CXCR1 intensity than the stromal areas (Figure 1E). Comparison of the CXCR1 immunostaining of the epithelial cells between eutopic endometrium of women with and without endometriosis, and ectopic endometriotic implants revealed an increasing expression from normal endometrial samples toward the eutopic, then to ectopic samples from women with endometriosis. Epithelial components of the ectopic endometriosis implants expressed significantly higher CXCR1 compared to their corresponding eutopic endometrium (*P* = 0.003) and normal endometrium (*P* < 0.001) (Figure 4). Fibrous tissues around the endometriotic foci were also strongly stained for CXCR1.

Epithelial CXCR2 expression was observed in both proliferative and secretory phases of all eutopic endometrial

### Figure 2.

Representative micrographs of immunohistochemistry staining for CXCR2 in the eutopic and ectopic endometrium of women with and without endometriosis. In both normal endometrial samples, epithelial staining was focal and weak, and mostly localized in the cytoplasm throughout the menstrual cycle (A, proliferative phase; B, secretory phase). Whereas, in eutopic endometrial samples of women with endometriosis, diffuse and strong immunostaining of CXCR2 was observed throughout the menstrual cycle (C, proliferative phase; D, secretory phase). Highest staining intensity in both epithelial and stromal components was observed in ectopic endometrial implants (E). Scale bars represent 50 μm (A, C and D); 80 μm (B); 200 μm (E).
samples of women with endometriosis, whereas stromal immunoreactivity was detected weakly in six samples from the proliferative and seven samples from the secretory phase of the cycle. In contrast to normal endometrial samples, epithelial CXCR2 immunostaining in eutopic endometrium of women with endometriosis was mostly diffuse and cytoplasmic rather than focal (Figure 2C and D), and the staining intensity was higher in both proliferative and secretory phase samples \((P < 0.05)\) (Figure 5). Myometrial compartment, vascular cells of arterioles, venules and capillaries showed weak staining in most samples, finding similar to normal endometrial samples.

On the other hand, similar to CXCR1 staining, all ectopic endometrial tissues expressed strong CXCR2 and this expression was most prominent in epithelial cells (Figure 2E). When we compared the epithelial HSCORE values of all endometrial samples from women with and without endometriosis, and ectopic endometriosis implants, both eutopic and ectopic endometrial epithelial CXCR2 HSCORE values were significantly higher than the normal endometrial epithelial values \((P < 0.05)\) (Figure 6). Fibrous tissues around the endometriotic foci were also stained strongly with CXCR2.

**Figure 3.** The distribution of CXCR1 immunostaining intensity (HSCORE) in epithelial cells of normal endometrium and eutopic endometrium of women with endometriosis according to the menstrual cycle phase. Data represent the mean HSCORE of samples of the menstrual cycle phase and error bars represent the SEM. In the eutopic endometrium of women with endometriosis, epithelial CXCR1 expression was higher throughout the menstrual cycle. In the proliferative phase, the epithelial CXCR1 expression was significantly higher than that of the normal endometrium, \(P < 0.05\).

**Figure 4.** The distribution of epithelial (proliferative and secretory) CXCR1 immunostaining intensity (HSCORE) between normal endometrium, eutopic endometrium and ectopic endometrial samples. A higher intensity in eutopic and ectopic endometrium was observed compared to normal endometrium samples, \(P < 0.001\).

**Figure 5.** The distribution of CXCR2 immunostaining intensity (HSCORE) in epithelial cells of normal endometrium and eutopic endometrium of women with endometriosis according to the menstrual cycle phase. Data represent the mean HSCORE of samples of the menstrual cycle phase and error bars represent the SEM. In the eutopic endometrium of women with endometriosis, epithelial CXCR2 expression was higher compared to normal endometrium samples in both proliferative and secretory phases, \(P < 0.05\).

**Figure 6.** The distribution of epithelial (proliferative and secretory) CXCR2 immunostaining intensity (HSCORE) between normal endometrium, eutopic endometrium and ectopic endometrial samples. Both eutopic and ectopic endometrial samples express higher levels of epithelial CXCR2 compared to that of normal endometrial samples, \(P < 0.05\).
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Discussion
The pathogenesis of endometriosis is still not completely understood despite intensive research on this topic. Since retrograde menstruation occurs in most of the reproductive age women, it is clear that there must be other factors which may contribute to the implantation of endometrial cells and their subsequent development into endometriotic disease (Gagne et al., 2003). Several studies revealed that endometriosis is associated with altered immune function, mainly observed in the peritoneal cavity (Harada et al., 2001; Lebovic et al., 2001; Berkkanoglu and Arici, 2003). The peritoneal environment of women affected by endometriosis contains several types of immune cells and their products (Harada et al., 2001; Lebovic et al., 2001; Seli et al., 2003). There is an increased number of activated macrophages in the peritoneal fluid of women with endometriosis, and these cells produce more cytokines and growth factors than that of normal women (Berkkanoglu and Arici 2003; Seli et al., 2003; Song et al., 2003). Although the total number, concentration and activation status of the peritoneal macrophages are increased in endometriosis, their capacity of eliminating ectopic endometrial cells is decreased compared to disease-free women (Braun et al., 1996). Moreover, it is postulated that, they may play a fundamental role in the establishment of the endometriotic disease by their secretory products, cytokines and growth factors (Garcia Velasco and Arici, 1999a; Seli et al., 2003).

IL-8 is a proinflammatory chemokine and is produced by several cell types such as macrophages, peritoneal mesothelial cells, trophoblasts and endometrial cells (Arici et al., 1996, 1998a). IL-8 induces chemotaxis of neutrophils and is also a potent angiogenic factor (Koch 1998; Arici et al., 1998a). IL-8 contributes in both physiologic and pathologic reproductive processes. We have shown previously the menstrual cycle-dependant changes in IL-8 mRNA and protein expression in the endometrium. The fact that the IL-8 mRNA levels are higher during the late secretory and early proliferative phases, lead us to speculate that IL-8 may modulate the timely recruitment of neutrophils and lymphocytes into the endometrium (Arici et al., 1998a). IL-8 is elevated in the peritoneal fluid of women with endometriosis and the levels correlate with the severity of the disease (Arici et al., 1996; Gazvani et al., 1998; Iwabe et al., 1998). We have previously reported that IL-8 stimulates the attachment of endometrial cells to the extracellular matrix, and this attachment stimulates further IL-8 production by these cells (Garcia Velasco and Arici, 1999b,c). Moreover it has been shown that this chemokine significantly stimulates cell proliferation in endometrial and endometriotic stromal cells (Arici et al., 1998b; Iwabe et al., 1998). In summary, IL-8 seems to act as an autocrine growth factor in the endometrium and may also play a role in the pathogenesis of endometriosis by promoting the vicious cycle of endometrial cell attachment, angiogenesis, cell growth, and further secretion of this cytokine in ectopic sites.

Chemokines exert their effect by binding to their membrane receptors, the expression of which may modulate their action. Two specific receptors for IL-8 have been identified on the surface of human neutrophils. The type A (CXCR1) binds with high affinity only to IL-8, but binds neutrophil-activating peptide-2 (NAP-2) and granulocyte-related oncogene-α (GROα) with low affinity. The type B (CXCR2) receptor binds the above three chemokines with high affinity (Ray and Samata, 1997).

Recently, Mulayim et al. investigated the expression of IL-8 receptors in normal endometrium. They found that epithelial and stromal CXCR1 expression shows menstrual cycle-dependent variation, with a peak expression during the mid secretory phase. On the other hand, they did not observe such variation for CXCR2 (Mulayim et al., 2002). In the present study the epithelial expression of both IL-8 receptors were similar throughout the menstrual cycle. Furthermore, we did not observe any stromal CXCR2 immunoreactivity in normal endometrial samples. In the present study, the use of paraffin-embedded sections in contrast to the previous study, in which frozen tissue sections were used, may explain the differences between the two studies.

IL-8 receptors show different characteristics of desensitization by their ligand. Compared to CXCR2, the affinity of CXCR1 for IL-8 is low, but its recovery rate is rapid after desensitization by IL-8. The rapid recovery rate of CXCR1 suggests that this receptor may play a more active role in mediating IL-8 signalling at the site of inflammation, where the IL-8 concentration would be high. On the other hand, high affinity of CXCR2 may initiate the neutrophil migration in a distant area of inflammation, where the concentration of IL-8 would be low (Chuntharapai and Kim, 1995). This information may potentially explain why in the former and in the present study, a much greater CXCR1 expression in comparison to CXCR2 was found in normal endometrium: as CXCR1 is expressed at a relatively higher level in normal endometrium, down-regulation of this receptor would be difficult, but its recovery would be rapid. Therefore, it becomes the predominant IL-8 receptor in normal endometrium. However, we observed that in the eutopic endometrium of women with endometriosis, both receptor expressions are increased, which reflects the inflammatory nature of this disease.

In the present study we report, to our knowledge for the first time, the protein expression of IL-8 chemokine receptors, CXCR1 and CXCR2, in eutopic and ectopic endometrial samples of women with endometriosis. Our data showed that, in the eutopic endometrium of women with endometriosis, epithelial and stromal IL-8 receptor expressions were increased throughout the menstrual cycle, compared to that of normal endometrium. Furthermore, ectopic implants expressed significantly higher levels of these receptors compared to normal endometrium. Since it is speculated that IL-8 may play fundamental roles in the establishment and maintenance of the ectopic endometrial cells in the peritoneal cavity, our results showing higher expression of IL-8 receptors in endometriosis support a potential role for IL-8 in the pathogenesis of endometriosis. Immunohistochemical staining technique is a semi quantitative method and it may limit the value of these findings. However, this method reveals the precise localization of the the receptors in eutopic and ecto-
pic endometrial tissues. Our study revealed that in normal endometrium, IL-8 receptors are expressed mainly in epithelial cells, similar to their ligand. On the other hand, in eutopic endometrium, expression of both receptors is also increased in stromal cells. This finding suggests that IL-8 may have more effect on the eutopic endometrium of women with endometriosis compared to normal endometrium.

Besides the effects of the peritoneal environment on the development of endometriotic disease, many authors nowadays believe that eutopic endometrium shares some alterations observed in the ectopic implants that are not found in the eutopic endometrium of healthy women. Recent studies revealed that the expression of many proteins (integrin αvβ3, aromatase enzyme, HOXA 10 and HOXA 11, cytokines and growth factors), which are involved in many reproductive processes such as menstruation, remodelling of endometrium, and preparation of the endometrium for blastocyst implantation, are differently expressed in eutopic endometrium of women with endometriosis compared to disease free woman (Lessey et al., 1994; Noble et al., 1996; Jolicoeur et al., 1998; Osuga et al., 1999; Taylor et al., 1999). In the present study, significantly higher epithelial expressions of CXCR2 in both eutopic and ectopic endometrium of women with endometriosis compared to normal endometrium indicates that the eutopic endometrium of women with endometriosis differ from normal endometrium. In addition, the trend for an increased epithelial CXCR1 expression from normal endometrium toward eutopic and ectopic endometrium may also indicate that there are differences between the two types of endometrium. Moreover, peritoneal environment may affect CXCR1 expression in ectopic sites. Our findings support the view that the primary defect in endometriosis may be in the eutopic endometrium of these women.

In conclusion, we report for the first time, the expression of IL-8 receptors, CXCR1 and CXCR2, in eutopic and ectopic endometrium of women with endometriosis. Our findings suggest that IL-8 and its receptor system are involved in the pathogenesis of endometriotic disease.

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