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Effects of 'casoparan', a peptide isolated from casein hydrolysates with mastoparan-like properties

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Casein, a protein found in milk of several species, is divided into different chains from 19 to 25 kDa. Casein is also considered as a source of amino acids and generating peptides with biological activities such as opiate, immunostimulating, antibacterial, peptidase inhibitors, among others.
In this work, Sephadex G-10 chromatography followed by high-performance liquid chromatography isolation purified NZCase TT, an industrial culture media for tetanus toxin production. In the first step, four pools were isolated and tested in different assays: isolated smooth muscle assay (guinea pig ileum, rat uterus), phagocytosis *in vitro* of opsonized sheep red blood cells, and hydrogen peroxide (H_2O_2) release from mouse peritoneal macrophages.
Pool III was the main active pool being able to potentiate bradykinin action in guinea pig ileum, stimulating phagocitic activity by resident macrophages and increasing H_2O_2 release from macrophages previously activated with bacille Calmette Guérin.
Using mass spectra the primary structure of the main peptide from pool III was obtained — INKKI, which corresponds to β-casein fragment 26–30.
The immunostimulating action is probably related to a direct action in macrophage cells.

Key words: Mastoparan, Casein peptides, Inflammation, Macrophages, Peptide isolation

Introduction

The proteolytic digestion of large proteins generates small bioactive peptides.[1] One of these proteins is casein and, besides its nutritional importance, revealed after tryptic and quimotryptic hydrolysis bioactive peptides able to regulate some important physiological pathways or acting in tissues, such as arterial blood pressure regulation, respiratory regulation, and in the immunological system among others.[2–4] These peptides can be generated after partial digestion and in some conditions could reach the blood stream, acting in different organs. This feature is particularly important in the newborn.[5] Peptides with opiate and bradykinin (BK)-potentiating effects were isolated.[3,6] Other important sources of bioactive peptides with high activity are found in venoms (snakes, spiders, scorpions) and in some insects such as wasp and ants.[7] These peptides have neurotoxic and peripheral effects including cellular and immune responses. Recently, a new class of peptides called mastoparans was isolated from wasps and bee venoms, and displayed actions on macrophages, hemolytic activity and edema.[8,9]

Macrophages are phagocytic cells known to play an important role in the immune response and in the development of the inflammatory process and defense reactions, participating in defense reactions against foreign cells and particles. Macrophage activation results in functional changes that lead to bactericidal or tumoricidal activities and the release of various enzymes, arachidonic acid metabolites and reactive forms of oxygen, as well as the synthesis of cytokines.[10,11]

Agents that show phagocytosis modulating activity and increase the functional capacity of the macrophage are of potential clinical significance. In this paper we describe the isolation and characterization of a new peptide derived from casein hydrolysates. We observed significant effects on rat arterial blood pressure and isolated guinea pig ileum, and in macrophages with an increase in phagocytosis and H_2O_2 liberation, showing that the isolated peptide from casein has a minimum structure that resembles mastoparan peptides obtained from wasp venoms.

Methods: purification of bioactive peptides from casein hydrolysates (NZCase)

Molecular ultrafiltration

Ten milliliters of a 10% solution (w/v) of NZCase in 0.02 M, pH 7.0 of sodium phosphate, was first

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filtrated with an Ultrafree-15 (Millipore, Bedford, MA, USA) molecular weight cut-off membrane of 5 kDa (this procedure was repeated six times). The filtrated material (8 ml) corresponding to 375 mg of the crude material was submitted to gel filtration chromatography in a Sephadex G-10 column (Amersham Biosciences, Uppsala, Sweden).

Sephadex G-10 gel filtration chromatography

The ultrafiltrated material (8 ml) corresponding to 375 mg of protein was applied to a Sephadex G-10 column (4×65 cm^2), previously equilibrated with sodium phosphate buffer solution (0.02 M, pH 7.0) and eluted with the same buffer with a 12 ml/h flow rate. Fractions of 3 ml were collected by a LKB-Pharmacia Redifrac fraction collector and each fraction was read in 280 nm in a Micronal Model B382 spectrophotometer (Micronal, São Paulo, Brazil). Fractions corresponding to peaks were kept together in four pools that were lyophilized and stored at -20°C. The protein of each pool was determined by the Lowry[12] method and compared with an albumin standard calibration curve.

High-performance liquid chromatography purification

After determination of the biological activity on isolated guinea pig ileum, pool III was submitted to high-performance liquid chromatography (HPLC) purification with an HP-1100 series liquid chromatograph coupled to an analytical reversed-phase C_{18} column (4.6 mm $\times$ 250 mm) Beckmann 5 µ ultrasphere ODS (Beckmann, San Ramon, CA, USA) using a linear gradient of acetonitrile in water–trifluoroacetic acid (0.1%) from 0–45% in 60 min to 45–90% in 30 min with a 1 ml/min flow rate, monitored at 214 nm. The main peaks were manually collected, lyophilized and stored at -20°C.

Peptide sequence

The HPLC-isolated active peak from pool III of the Sephadex G-10 chromatography was sequenced by Edman degradation in a Shimadzu sequencer apparatus model PSPQ-1 (Shimadzu, Kioto, Japan) and the sequence was confirmed by amino acid analysis following the method previously described by Heiriksson and Meredith,[13] and also by mass spectra in a triple quadrupole Micromass mass spectrometer model Quatro II (Micromass, Milford, MA, USA) by analyzing the mass weight spectra of the generated peptides and amino acids.

Peptide synthesis

After primary sequence determination of the peptide, the synthetic peptide was obtained by liquid phase synthesis, and the identity of the effects obtained with the natural and synthetic peptide was checked as described previously by Juliano and Juliano.[14]

Peritoneal cell preparation

Animals were anesthetized with ether and killed through exsanguination by sectioning the cervical vessels. The peritoneal cavity was washed three times with cold phosphate-buffered saline (PBS), pH 7.4. After a gentle massage of the abdominal wall, the peritoneal fluid, containing resident macrophages, was collected. Elicited macrophages were obtained by peritoneal wash 4 days after intraperitoneal injection of thioglycollate broth (4%). Activated cells were obtained by peritoneal washing 10 days after injection (intraperitoneally) of 2 mg of heat-inactivated ONCO-BCG (bacille Calmette Guérin; Butantan Institute, São Paulo, Brazil). Cell viability was assessed by the trypan blue exclusion test ($>95\%$). Total peritoneal cells were determined in a Neubauer's chamber, and the differential counts were performed in smears stained with Wright and May Giemsa modified stain methods. For all measurements, samples of individual animals were used.

Phagocytic activity of peritoneal macrophages

A suspension of sheep erythrocytes was diluted in PBS (0.5%) and mixed (v/v) with rabbit antiserum against sheep erythrocyte; the mixture was then incubated for 30 min at 37°C. The opsonized erythrocytes were centrifuged twice, at $184 \times g$ for 10 min, with PBS and suspended with RPMI 1640 medium (0.5%) for the phagocytosis assay. Briefly, the coverslips containing the adherent and spreading of peritoneal resident or elicited macrophages[15] were incubated with 1 ml of RPMI 1640 medium containing opsonized sheep erythrocytes, for 40 min, at 37°C, in an atmosphere containing 5% CO_2. The percentage of phagocytosis was determined by examination under a phase contrast microscope, by counting the percentage of cells that had phagocytosed more than five particles.

Effect of the peptide on phagocytic activity: dose–response curve

The effect of peptide over phagocytic activity of resident peritoneal macrophages was assessed *in vitro*. The cover slips containing adhered macrophages were incubated with RPMI 1640 medium (control) or RPMI 1640 containing the peptide (12.5, 25, 50 and 100 µg/ml) at 37°C, in an atmosphere

containing 5% CO_2. After 1 h, the coverslips were washed with PBS and prepared for the phagocytosis assay.

Hydrogen peroxide production

Hydrogen peroxide production (H_2O_2) was measured as described by Pick and Mizel[16] and modified by Russo *et al*.[17] Briefly, the peritoneal activated cells were adjusted to $1.5-2.5 \times 10^6$ cells/ml and centrifuged for 10 min at 1000 rpm and 5°C. The sediment was resuspended in 1 ml of phenol red solution, containing 140 mM NaCl, 10 mM potassium phosphate buffer, pH 7.0, with 0.5 mM dextrose, 0.28 mM phenol red (PRS), and 8.5 U/ml of horseradish peroxidase for H_2O_2 detection. The final volume of 7.4 ml was obtained with Hank's solution. One hundred microliters of the cell suspension were plated onto each well of 96-well flat-bottomed tissue culture plates (Corning, New York, USA) and 10 µl of the peptide (5.0 or 10.0 µg) or saline was added and incubated in a humidified atmosphere at 37°C for 1 h. Vertical row number 1 was left without cells and filled with 100 µl per well of PRS. The second and third verticals rows were used for the establishment of H_2O_2 standard curves. These wells were covered with 100 µl of PRS, added to 10 µl of H_2O_2 solution, resulting in a final concentration of H_2O_2 ranging from 5 to 40 µM.

The subsequent rows contained wells covered with 100 µl of PRS in the absence (basal H_2O_2 production) or the presence of phorbol myristate acetate (PMA) (20 ng). After 60 min incubation at 37°C, the reaction was stopped by the addition of 10 µl of 1 N NaOH solution. H_2O_2-dependent phenol red oxidation was measured spectrophotometrically at 620 nm, in a Labsystem Multiscan apparatus (Labsystem, Helsinki, Finland). The concentration of H_2O_2 was calculated from absorbance measurements, as described by Pick and Mizel[16] and expressed as nmoles of H_2O_2 per milliliter per 2.5×10^5 cells using the linear regression calibration curve for each assay.

Isolated guinea pig ileum and isolated rat uterus assays

The terminal piece of the isolated guinea pig ileum and the rat uterus (from animals injected 18 h previously with 1 mg/kg of diethylstilbestrol) were used. The organs were suspended in a 5 ml chamber filled with Tyrode's solution (138 mM NaCl, 2.7 mM KCl, 1.05 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.42 mM $NaHPO_4$, 11.9 mM $NaHCO_3$, and 5.55 mM glucose) containing atropine sulphate and diphenydramine (1 mg/ml) at 35°C for the isolated guinea pig ileum, and Jalon solution (154 mM NaCl, 5.63 mM KCl, 0.54 mM $CaCl_2$, 5.95 mM $NaHCO_3$ and 2.8 mM glucose) maintained at 29°C for the uterus. The solutions were bubbled with air, and the muscle contractions were recorded on a polygraph coupled to an isotonic transducer. The substances tested were preincubated with the smooth muscle for 1 min. The BK potentiating effect of '*in vitro*' and '*in vivo*' experiments were calculated as described previously by Lebrun *et al*.[3] One potentiation unit represents a twofold increase of ileum or uterus contraction for a constant BK dose. The standard error for these values was less than 5%.

Statistical analysis

Statistical analysis was performed using analysis of variation test and the Student *t*-test and the Student–Newmann–Keuls test by means of the Primer software for statistical analysis first version and the $p < 0.05$ significance level was adopted.

Results

From a 10% solution of 8 g of casein digest (NZCase) previously submitted to molecular ultrafiltration in a 5 kDa Ultrafree membrane, resulting in a 2 g filtrate that was applied to a Sephadex G-10 gel filtration chromatography column (4×65 cm) with a 12 ml/h flow rate, fractions of 3 ml were collected and the absorption was determined at 280 nm, resulting in four main peaks (pool I, pool II, pool III and pool IV) (Fig. 1). The four pools were tested and the active pool III was the submitted to HPLC chromatography (Fig. 2), resulting in an isolated peak that was sequenced.

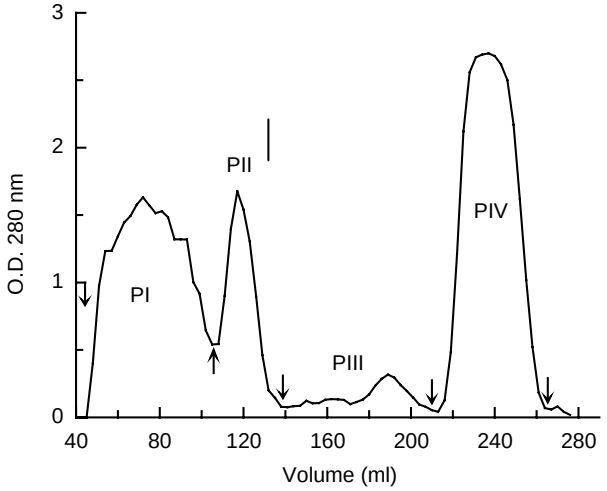

FIG. 1. Purification and isolation of casoparan peptide INKKI from casein hydrolysates (NZCASE). (A) Gel filtration chromatography purification of NZCase after ultrafiltration in a 5000 Da membrane in a Sephadex G-10 column (4×65 cm^2) eluted with 0.02 M Na_2PO_4, pH 7.0, solution, monitored at 280 nm, flow rate of 12 ml/h and 3 ml of each fraction. Pool III (PIII) showed actitivity and was purified by HPLC.

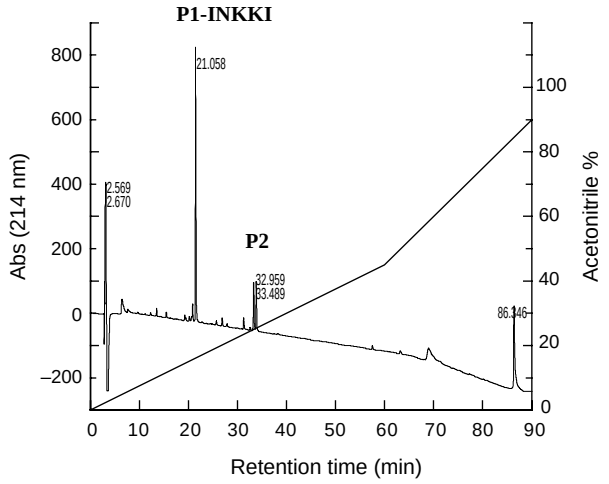

FIG. 2. HPLC chromatography of pool III obtained from Sephadex G-10 chromatography in a C_{18} analytical reversed-phase column eluted with a linear gradient of acetonitritrile 0–45% in 45 min and 45–90% acetonitrile in 45–70 min in TFA 0.1% (in water), flow rate of 1 ml/min monitored at 214 nm.

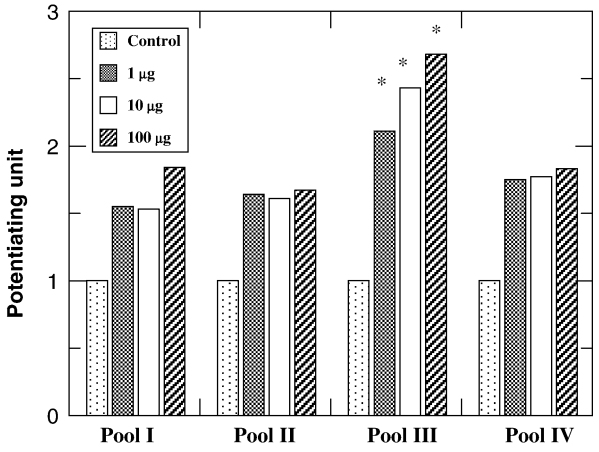

FIG. 3. BK-potentiating activity obtained with the isolated pools (I, II, III and IV) from a Sephadex G-10 column in the isolated guinea pig ileum. The potentiating-activity (PU) was calculated as described in Methods. Only pool III showed significant differences when compared with controls (Student *t*-test, $*p < 0.05$).

Mass spectra analysis

After mass spectra of the HPLC-isolated peptide and analysis with the MassLynx and MaxEnt software, it was possible to obtain the sequence of the peptide INKKI that corresponds to the 26–30 fragment of the bovine beta-casein. The sequence was confirmed by sequencing in a PSPQ-1 Shimadzu sequencer by Edman degradation. The biological effects of the natural isolated peptide were confirmed using the synthetic peptide obtained by liquid phase synthesis, as described previously by Juliano and Juliano.[14]

Biological assays

Isolated guinea pig ileum assay was performed with the pools obtained from Sephadex G-10 gel filtration chromatography. The pools obtained from Sephadex G-10 gel filtration chromatography were tested on the isolated guinea pig ileum to observe the possible contractile effects or the BK-potentiating activity was determined as potentiating unity as described in Methods.

The same effect was observed using the isolated rat uterus and no intrinsic effect was observed with the two isolated smooth muscle preparations. Pool II revealed a dose–response relationship in potentiation of BK effects on isolated guinea pig ileum (Fig. 3), and from this pool the peptide INKKI was isolated as the main component by HPLC chromatography (Fig. 2).

Besides the potentiating effects on BK, the action of the isolated peptide INKKI isolated after HPLC chromatography on macrophage functions was investigated. A dose–response relatioship was observed on macrophage erythrocyte phagocytosis with doses ranging from 10 to 100 µg/ml, but over 30 µg/ml a plateau is reached (Table 1). The peptide INKKI caused increase on phagocytosis by resident macrophage when compared with the control (saline). The concentration of the 25 µg/ml induced the higher increase (80%) in the resident macrophage, suggesting that the peptide stimulates the phagocytic activities (Table 1).

No significant difference was observed when 25 µg of the INKKI peptide was assessed *in vitro* on the phagocytic activity of the resident peritoneal macrophages; but the INKKI peptide showed a significant stimulatory effect on resident macrophages (Fig. 4).

The 5 or 10 µg of INKKI caused an increase in the stimulated by PMA (38%, 173%) and unstimulated (30%, 38%) production of H_2O_2 of peritoneal activated macrophage (Fig. 5A,B).

Discussion

Phagocytosis is a process used by mononuclear phagocytes to ingest and clear large particles, including infectious agents, senescent cells and cellular debris.[18] Particle internalization is started by the interaction of specific receptors on the surface of

Table 1. Dose–response activity of casoparan peptide (INKKI) on phagocytic activity of resident rat peritoneal macrophages

Dose (µg/ml)	Stimulation (%)
12.5	17
25	80
50	67
100	47

Results are expressed as percentage of controls.

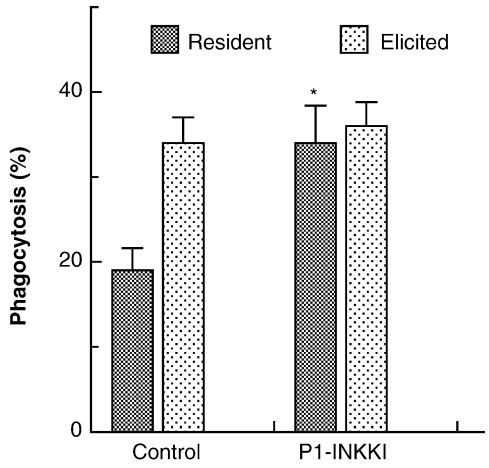

FIG. 4. Phagocytic activities of resident (dark shading) or elicited (light shading) peritoneal macrophages incubated with casoparan peptide INKKI (25 μg/ml) or saline (control group). The results are expressed as the mean ± standard error of the mean for 8–10 animals per group. $*p < 0.01$, significantly different from the control group.

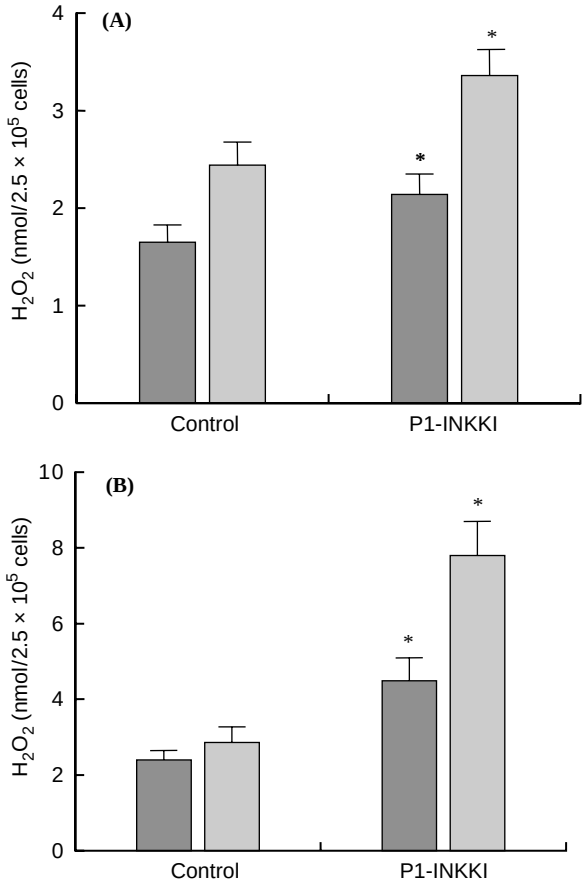

FIG. 5. Effects of the casoparan peptide INKKI, 5 μg (A) and 10 μg (B), on H_2O_2 production by peritoneal macrophages. The cells were obtained from the peritoneal cavity of mice injected 10 days before the experiments with BCG. H_2O_2 (nmol H_2O_2/2.5 × 10^5 cells/ml) was determined in the absence (dark shading) or in the presence of PMA (light shading). The results are expressed as the mean ± standard error of the mean for 8–10 animals per group. $*p < 0.05$, significantly different in comparison between H_2O_2 production in the presence of PMA and in the absence of PMA.

the phagocyte with binding on the surface of the particle.[19,20]

Several studies have demonstrated the effects of peptides on the phagocytic activities of macrophages.[5,21,22]

Data presented herein showed that the peptide INKKI from HPLC isolation of pool III stimulates phagocytic activities of resident peritoneal macrophages *in vitro* mediated by the Fc receptor. Resident macrophages occur in specific sites in normal and non-inflamed tissues. Elicited macrophages are cells attracted to a given site because of a particle stimulus, independent of the developmental stage or functional state of the cells, and activated macrophages are cells with increased or new functional activities induced by a given stimulus.[23]

The results showed that the stimulant effects of the peptide depend on the cell activation stage since they are only observed on resident macrophages, which are quiescent cells. These cells are responsive to inflammatory or immunological signals. The increase of the phagocytosis observed in the resident cells was similar to the effects observed on elicited macrophages, suggesting that the peptide could act as a macrophage stimulation inducer. This stimulatory effect was dose dependent and 80% stimulation was observed in the presence of the casoparan peptide INKKI 25 μg/ml, while high doses induced a minor response, suggesting a possible saturation of the phenomena. Similar results were obtained when the synthetic peptides were used to assay the phagocytic activities.

The phagocytosis process stimulates the respiratory burst.[24] The generation of reactive oxygen intermediates such as superoxide and H_2O_2 is a practical measuring system for the respiratory burst;[25] and the reactive oxygen intermediates take part in the microbicidal mechanism used by macrophages and neutrophils.[20] The peptide INKKI stimulated the production of hydrogen peroxide only in macrophages activated by BCG; this effect was observed in the presence or absence of PMA. However, the presence of PMA increases this production. Among the soluble stimuli, only PMA induces a marked respiratory burst with an abundant release of H_2O_2.[25] The results showed that the peptide as well as the PMA induce the respiratory burst. The PMA stimulates the oxidative burst, bypassing the need for receptor–ligand coupling stimulating a Ca^{2+}-dependent protein kinase C.[26,27] Therefore, the mechanism of the peptide actions remains unknown. Previous publications report that some peptide fragments of casein show immunostimulating or immunomodulator effects.[28–30] A hexapeptide corresponding to the 54–59 fragment of human β-casein (VEPIPY) showed a stronger immunostimulating effect and conferred resistence in experimental infections with *Klebsiella pneumoniae*.[31] Moreover,

peptides isolated from wasp, bee and ant venoms, called Mastoparans, exhibit inflammatory properties, releasing histamine, provoking edema and cell migration to the inflammatory site. These peptides are approximately 1400 Da and have a structure rich in Ile, Asn and Lys.[32] The isolated pentapeptide INKKI showed the same motif present in the mastoparans less the neutral amino acids (Ala, Gli and Leu). The biological effects include an increase of phagocytosis and H_2O_2 production. These effects happen even in the presence of PMA, suggesting a specific pathway for the peptide action. The peptide INKKI also induced histamine release in rat arterial blood pressure and isolated guinea pig ileum, since the effect is blocked by diphenydramine (data not shown).

The effects displayed by casoparan peptide suggest that the generation of small peptides from large proteins with physiological functions is particularly important. In this particular feature, the peptide isolated from casein could lead to modulatory effects over inflammatory cells such as macrophages, increasing phagocytosis and H_2O_2 liberation without the development of inflammation. This aspect considering the physiological generation in the newborn could be an adaptive stimulus to the immature immunological system that is on development.

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