Protein-energy malnutrition alters the C3 complement factor in response to lipopolysaccharide in a murine model

A desnutrição proteico-energética altera a concentração do fator C3 do sistema complemento em resposta ao lipopolissacarídeo em um modelo murínico

ABSTRACT

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Malnutrition modifies the host's resistance to infection, altering many physiological processes, including hematopoiesis and immunological functions. In this study, we evaluated the total complement system and its C3 component factor in animals subjected to a protein malnutrition model with lipopolysaccharide (LPS) stimulation. The cellularity of blood, bone marrow and spleen, as well as the production of C3 and CH50, were evaluated. Two-month old male Swiss mice were submitted to protein malnutrition with a low-protein diet containing 4% protein. A control group received a control diet containing 20% protein. When the experimental group had reached a loss of about 20% in their original body mass, they were intravenously inoculated with LPS. Cells in the blood, bone marrow and spleen were counted and the circulating levels of C3 and CH50 were evaluated in these animals. Malnourished animals presented anemia, leucopenia, and a severe reduction in bone marrow and spleen cellularity. The survival rate of the malnourished animals was lower, as well as the production of C3 and CH50, if compared to the control animals. These findings suggest that malnourished mice present a deficient response to LPS. The decrease in the complement synthesis may be partially responsible for the immunodeficiency observed. These data lead us to conclude that the nutritional status interferes in the immune response activation.

Keywords: Malnutrition. Complement system. C3 complement factor.

RESUMEN

La desnutrición proteico-energética modifica la resistencia a la infección, alterando numerosos procesos fisiológicos, incluyendo la hematopoyesis y la función inmunológica. En este estudio medimos las concentraciones séricas del factor C3 y del Sistema Complemento Total (CH50) en ratos con desnutrición proteico-energética estimulados con lipopolisacárido (LPS). Fue evaluada la celularidad periférica, medular y esplénica. Ratos Swiss, machos, adultos, con dos meses de edad fueron sometidos a desnutrición proteica con una dieta de 4% de proteína .El grupo control consumía una dieta con 20% de proteínas. Cuando los animales desnutridos perdieron 20% de su peso inicial, fueron inoculados por vía endovenosa con LPS. Fueron determinadas las concentraciones de células sanguíneas, de la médula ósea, del bazo y los valores de C3 y CH50 circulantes en los animales estimulados. Los animales desnutridos presentaron anemia y leucopenia además de una reducción significativa de celularidad de la médula ósea y del bazo. La sobrevivencia de este grupo era menor y también eran más bajas las concentraciones del factor C3 del complemento y del complemento total, en relación a los animales del grupo control. Los resultados sugieren que animales desnutridos muestran una respuesta deficiente a LPS. La síntesis menor de complemento puede ser en parte responsable por la inmunodeficiencia observada. Estos resultados nos conducen a inferir que la desnutrición proteico-energética interfiere en la activación de la respuesta inmune.

**Palabras clave: Desnutrición.
Sistema complemento.
Factor C3 del complemento.**

RESUMO

A desnutrição proteico-energética modifica a resistência à infecção, modificando diversos processos fisiológicos, incluindo a hematopoiese e as funções imunológicas. Neste estudo, avaliamos as concentrações séricas do fator C3 e do Sistema Complemento total (CH50) em um modelo no qual camundongos submetidos à desnutrição proteico-energética são estimulados com lipopolissacarídeo (LPS), e avaliamos a celularidade periférica, medular e esplênica. Camundongos Swiss, machos, adultos, com dois meses de idade foram submetidos ao processo de desnutrição proteica com uma dieta contendo 4% de proteína em comparação aos animais controles com uma dieta contendo 20% de proteína. Quando os animais do grupo desnutrido alcançaram aproximadamente 20% de perda de peso, em relação ao inicial, foram inoculados endovenosamente com LPS. As células do sangue, da medula óssea e do baço foram quantificadas, bem como as concentrações circulantes de C3 e CH50 em animais estimulados com LPS. Os animais desnutridos apresentaram anemia e leucopenia, além de redução significativa da celularidade da medula óssea e do baço. Os animais desnutridos apresentaram menor taxa de sobrevivência, bem como das concentrações do fator C3 do complemento e do complemento total em relação aos animais controles. Estes resultados sugerem que animais desnutridos apresentam uma resposta deficiente aos LPS. A síntese menor do complemento pode ser em parte responsável pela imunodeficiência observada. Estes resultados conduzem-nos a inferir que a desnutrição proteico-energética interfere na ativação da resposta imune.

**Palavras-chave: Desnutrição.
Sistema Complemento.
Fator C3 do Complemento.**

INTRODUCTION

Protein-energy malnutrition (PEM) is the most frequent type of malnutrition. It occurs especially in children, the elderly, patients suffering from neoplasia or chronic illnesses or undergoing chemotherapy (DE ONIS et al., 1993; RICE et al., 2000).

Several different studies have demonstrated that malnourished individuals present an increased susceptibility to infections (GROSS; NEWBERNE, 1980; KEUSCH, 2003; NOVA et al., 2002). Malnutrition alters many physiological processes, including hematopoiesis and the immune response, which brings to evidence the close interaction between malnutrition and infection. PEM modifies the specific and nonspecific immune response of the organism (CHANDRA, 1992; GROSS; NEWBERNE, 1980; REDMOND et al., 1991) by the decreased production of hematopoietic cells, resulting, eventually, in bone marrow hypoplasia (BORELLI; MARIANO; BOROJEVIC, 1995; ORTIZ; BETANCOURT, 1984; VITURI et al., 2001; XAVIER et al., 2007), it also induces structural alterations in lymphoid organs and thymus-dependent areas (MUNOZ; MARCOS; UNZAGA, 1981) which could interfere in the animals' resistance to infections. The exact mechanisms by which malnutrition affects the immune response are not fully understood yet.

The recognition of pathogen surface molecules, such as surface carbohydrates and lipopolysaccharides (LPS) is the first step in the immune response. The complement system is an effector mechanism of humoral immunity, innate and acquired, that plays an important role in the defense of the organism against infections (LISZEWSKI; ATKINSON, 1998).

Complement proteins raise interest because they seem to be a key component of the innate immune system as well as participate in the adaptive immunity phylogenetic development (MICHAEL; FRANK, 2000).

The complement cascade consists of 3 separate pathways that converge into a final common one. The pathways are: the classic pathway (C1qrs, C2, C4), the alternative pathway (C3, factor B, properdin), and the mannan-binding (mannan-binding lectin, MBL) pathway. The terminal complement consists of all proteins activated after C3, forming a group of proteins, known collectively as the membrane attack complex (MAC). MAC carries out effective killing activity by creating perforations in cellular membranes (BOHLSON et al., 2001). Deficiencies in complement components predispose patients to infection via two mechanisms (LISZEWSKI; ATKINSON, 1998): ineffective opsonization and defects in lytic activity (defects in the MAC). Specific complement deficiencies are also associated with an increased risk of developing autoimmune diseases such as systemic lupus erythematosus (BOHLSON et al., 2001; MICHAEL; FRANK, 2000).

In this study, we evaluated total complement (CH50) and its C3 component in animals submitted to PEM stimulated with LPS.

MATERIALS AND METHODS

DIETS

The murine diets were prepared in our laboratories. Two diets were prepared: one for the malnourished group animals and another for the control group animals. Both diets contained fibers, saline mixtures and balanced vitamin mixtures in the same quantity; although the control diet contained 20% protein while the low-protein purified diet contained only 4% protein, (BORELLI; MARIANO; BOROJEVIC, 1995; FRIED et al., 1978). Casein was used as the protein source. Except for the protein content, both diets were identical and isocaloric (Table 1). The final protein content of each diet was monitored by the standard micro-Kjeldahl method (WARD, 1963).

Table 1 - Experimental diet composition[1]

Ingredients	Control Diet	Malnourished Diet
	(g/kg diet)	
Casein (>85% protein)	200	40
Sucrose	100	100
Fiber	10	10
Corn oil	80	80
Mineral mixture[2]	40	40
Vitamin mixture[2]	10	10
L-Methionine	1.5	1.5
Choline bitartrate	2.5	2.5
Corn starch	556.5	716.5

[1]Isocaloric diets providing 1716.3 kJ/100g (410.6kcal/100g).

[2]Mineral and vitamin mixtures were prepared according to the 1993 recommendations of the American Institute of Nutrition for adult mice (REEVES et al. 1993).

ANIMALS

Male outbred 2-3-month-old Swiss mice were obtained from the Animal Laboratory of the Faculty of Pharmaceutical Sciences at the University of São Paulo. They were placed in individual wire-mesh cages, and received the control diet for 21 days. After this period of adaptation, they were subsequently separated into four groups: Control and Malnourished, both with LPS or without LPS (C, C+LPS, M and M+LPS), maintained under regular light/dark cycle of 12h and in a temperature of 22°-25°C and humidity of 55% ± 10%, receiving either the control or the low-protein diet and water *ad libitum*. Their body weight and the consumption

of food were monitored every 48h. The mice were submitted to experimental assays after 14 days eating their respective chows, when the undernourished group reached a loss of about 20% in their original body weight (BORELLI; MARIANO; BOROJEVIC, 1995). For the collection of different biological, the animals were previously anesthetized with xylazene hydrochloride (Rompum®, Bayer, 10mg/Kg) and with ketamide hydrochloride (Ketamina®, Cristália, 100mg/Kg). All procedures were carried out under aseptic conditions. The reagents and materials used were pyrogen-free. This study was approved by the Commission on Ethics in Animal Studies of the Faculty of Pharmaceutical Sciences at the University of São Paulo, Brazil, according to the Brazilian College on Animal Experimentation guidelines.

LPS ENDOTOXEMIA

Escherichia coli (055:B5) lipopolisaccharide (LPS) (Sigma Chemical Company, St. Louis, MO) was diluted in pyrogen-free isotonic saline solution. Animals in the C+LPS and M+LPS groups received 1.25µg of LPS intravenously.

NUTRITIONAL EVALUATION

A nutritional evaluation of each animal was performed by measuring body weight, diet consumption and the plasma concentrations of protein, albumin and pre-albumin.

BLOOD CELLS AND PLASMA PROTEINS

Mice were anesthetized and the whole blood samples were obtained by cardiac puncture and collected concomitantly with bone marrow and spleen cells. The blood samples were collected in tubes containing EDTA as anticoagulant in a final concentration of 1mg/mL of blood (EDTA, Sigma Chemical Company, St. Louis, MO). The hemoglobin concentration and hematocrit were determined by standard methods (DACIE; LEWIS, 1995). Total cell counts were obtained using a Neubauer chamber. Differential leukocyte counts were performed on blood smears stained by the standard May-Grunwald and Giemsa solutions (Sigma Chemical Co., St. Louis, MO).

Plasma was separated by centrifugation and the total protein content, albumin and pre-albumin were determined by standard methods used in clinical laboratory tests (DOUMAS; WATSON; BIGGS, 1971; GORNALL; BARDAWILL; DAVID, 1949). Blood collected without anticoagulant was obtained from all groups. The serum was separated by centrifugation (2000 x g for 10 minutes, 4°C) and frozen in aliquots at -40°C until analysis.

BONE MARROW AND SPLENIC CELLULARITY

Bone marrow cells were obtained by flushing the femoral cavity with 1mL of McCoy's 5A medium (Sigma, Chemical Company, USA). The spleen was removed, placed in Petri

dishes containing 5mL of McCoy's 5A medium with EDTA (1mg/mL) and dissociated gently using needles and tweezers. The number of total cells was quantified in a standard hemocytometer.

C3 AND CH50 DETERMINATION

The concentrations of CH50 and the C3 complement component were measured by using the commercially available kits (N Antisera Complement Factors, Dade Behring®, Marburg, Germany) for immunonephelometry in the BN system (Dade Behring®, Marburg, Germany). The concentrations of CH50 and C3 were determined in serum, which was separated from venous blood obtained from control and malnourished animals.

STATISTICAL ANALYSIS

Results are expressed as means ± standard deviations of the means (SD). Data were analysed using one-way analyses of variance and Tukey's Multiple Comparison Test (p≤0.05) with the aid of computer software GraphPad Prism®. Survival analyses between groups was calculated using the log rank method.

RESULTS

PROTEIN CONSUMPTION, BODY WEIGHT AND PROTEIN, ALBUMIN AND PRE-ALBUMIN PLASMA CONCENTRATIONS

Mice maintained on the low-protein diet spontaneously reduced their food consumption compared to control mice, which resulted in the reduction of protein consumption, body weight loss and decrease in protein, albumin and pre-albumin plasma concentrations (Table 2). The control group received an adequate protein diet and the malnourished group received a protein-deficient diet. The malnourished group presented a markedly lower feed intake and experienced a 22.1% loss of body weight in a period of 14 to 16 days after the introduction of the low-protein diet.

SURVIVAL WITH LPS

A significantly lower survival rate (83%, p ≤0.05) was observed in malnourished animals 24 hours after the inflammatory stimulus with LPS, when compared to the control group (99%).

BLOOD

The malnourished animals of both groups (M and M+LPS) presented anemia with a decrease in hemoglobin concentration, hematocrit, total number of erythrocytes and

leucocytes (polymorphonuclear leucocytes, lymphocytes and monocytes were all found in lower quantities in these animals than in the control animals) (Table 3).

Table 2 - Diet and protein consumption, body weight variation and total protein, albumin and pre-albumin concentrations

Groups	Control (n=8) Whitout LPS	Control (n=8) LPS 24 hours	Malnourished (n=8) Whitout LPS	Malnourished (n=8) LPS 24 hours
Diet consumption (g/day/animal)	6.52 ± 0.29[a]	6.11 ± 0.33[b]	4.06 ± 0.18[a, b]	3.99± 0.22[a, b]
Protein consumption (g/day/animal)	1.31± 0.14[a]	1.23 ± 0.12[b]	0.16 ± 0.05[a, b]	0.16 ± 0.07[a, b]
Body weight variation (%)	4.19 ± 0.8[a]	4.33 ± 0.4[b]	-22.1 ± 1.4[a, b]	-23.0 ± 1.9[a, b]
Plasma protein (g/dL)	7.2 ± 0.98[a]	7.4 ± 0.33[b]	4.8 ± 0.81[* a, b]	4.9 ± 0.77[a, b]
Albumin (g/dL)	4.2 ± 0.6[a]	4.2 ± 0.7[b]	3.1 ± 0.4[a, b]	3.0 ± 0.5[a, b]
Pre-albumin (mg/dL)	10.8 ± 1.2[a]	10.9 ± 1.9[b]	2.9 ± 0.81[a, b]	2.7 ± 0.77[a, b]

Values expressed as mean ± standard deviation (SD). The number in parentheses denotes the number of animals used in the experiment. [a]indicates significant difference between the control group without LPS and other groups (p ≤0.05). [b]indicates significant difference between the control group with LPS and other groups (p ≤0.05).

Table 3 - Erythrocytes, hemoglobin, hematocrit, leucocytes, polymorphouclear, lymphocytes and monocytes values

| Groups	Control (n=8) Whitout LPS	Control (n=8) LPS 24 hours	Malnourished (n=8) Whitout LPS	Malnourished (n=8) LPS 24 hours
Erythrocytes (x 10^6/mm³)	7.52 ±0.21[a]	7.82 ± 0.33[b]	4.08 ± 0.15[a,b]	3.99 ± 0.33[a,b]
Hemoglobin (g/dL)	13.3 ± 0.6[a]	13.9 ± 0.8[b]	6.6 ± 0.9[a,b]	5.8 ± 0.21[a,b]
Hematocrit (%)	36.3 ± 1.55[a]	37.1 ± 1.11[b]	19.8 ± 2.85[* a,b]	17.9 ± 1.29[a,b]
Leucocytes (/mm³)	3433 ± 211.5[a,b]	4210 ± 121.1[b]	1470 ± 65.7[a,b]	1125 ± 90.5[a,b]
Polymorphonuclear granulocytes (/mm³)	602 ± 14.1[a]	823 ± 92[b]	186.7 ± 4.3[a,b]	200± 4,3[a,b]
Lymphocytes (/mm³)	2399 ± 45.0[a]	2410 ± 127[b]	1370 ± 40.0[a,b]	987± 43[a,b]
Monocytes (/mm³)	75.8 ± 2.2[a]	14.7± 0.33[a,b]	10.1± 0.5[a,b,c]	2,0 ± 0,22[a,b,c]
Bone Marrow Total Cells (x10⁷/mL)	0.87 ± 0.07[a]	0.82 ± 0.24[b]	0.58 ± 0.04[* a,b]	0.51± 0.11[a,b]
Spleen Total Cells (x10⁷/mL)	0.83 ± 0.07[a]	0.78 ± 0.22[b]	0.49 ± 0.05[a,b]	0.44 ± 0.42[a,b]

Values expressed as mean ± standard deviation (SD). The number in parentheses denotes the number of animals used in the experiment. [a]indicates significant difference between the control group without LPS and other groups (p ≤0.05). [b]indicates significant difference between the control group with LPS and other groups (p ≤0.05). [c]indicates significant difference between the malnourished group without LPS and malnourished with LPS (p ≤0.05).

BONE MARROW AND SPLEEN CELLS

Animals of the malnourished groups (M and M+LPS) presented a decrease in the number of blood cells in bone marrow and spleen (Table 3). The granulocytic to erythroid (G/E) ratio in the malnourished animals was significantly lower ($p \leq 0.05$) than in the control animals (data not show).

DETERMINATION OF C3 AND CH50

Malnourished animals, without stimulus, presented lower concentrations of C3 when compared to control animals. After the induction of the inflammatory process with LPS, control animals presented higher total complement (CH50) and C3 component concentrations when compared with others groups (Figure 1).
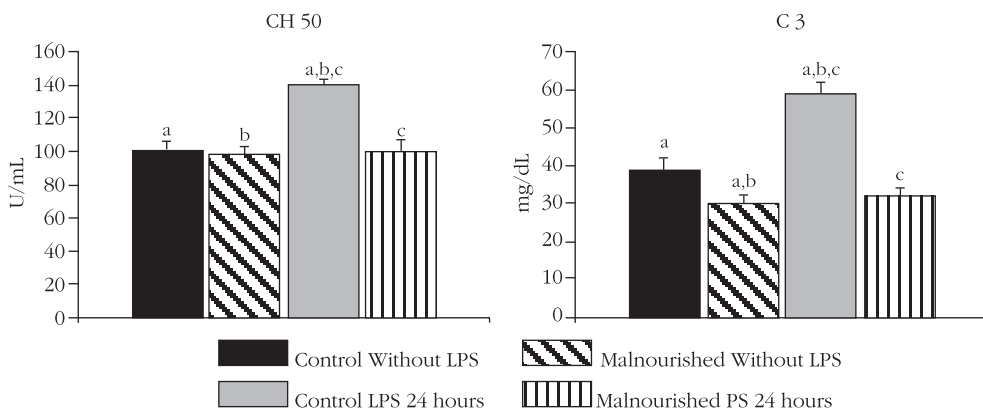
Values expressed as mean ± standard deviation (SD). Control animals (n=8), Control animals with LPS (n=8), malnourished animals (n=8) and malnourished animals with LPS (n=8). [a] indicates significant difference between the control group without LPS and other groups ($p \leq 0.05$). [b] indicates significant difference between the control group with LPS and other groups ($p \leq 0.05$). [c] indicates significant difference between the malnourished group without LPS and malnourished with LPS ($p \leq 0.05$).

Figure 1 - CH50 and C3 serum concentrations

DISCUSSION

Alterations in diet and nutritional status influence the inflammatory response and the efficacy of response to infections (CHANDRA, 1999; MARTORELL, 1999). Other studies by our group have demonstrated that animals maintained on a low-protein diet present alterations in their specific and nonspecific mechanisms of defense against infection,

including hemopoiesis, celularity and structure of lymphoid organs and thymus-dependant areas and modifications in some functional aspects of phagocytic cells such as the respiratory burst and the ability of these cells in synthesizing nitric oxide and pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) (BORELLI; MARIANO; BOROJEVIC, 1995; BORELLI et al., 1998; FOCK; SILVA; BORELLI, 2003; FOCK et al., 2007; VITURI et al., 2001).

The control group received a diet with adequate protein content whereas the test group received a protein-deficient diet. The deficient group presented a markedly lower feed intake and experienced a loss of about 20% of their body mass. Despite malnourished groups ingested a lower amount of feed, their diet met the minimum nutrient requirements for adult mice except for protein (REEVES; NIELSEN; FAHEY, 1993). Since the minimum daily amounts of nutrients other than protein were ingested by the animals of the malnourished group, we can conclude that the changes observed in our experimental model are mainly the result of the decrease in protein and energy intake.

Data found in literature as well as our own data also indicate that in situations when malnutrition is not associated with other diseases, leucopenia always occurs (BORELLI et al., 1998; CATCHATOURIAN; ECKERLING; FRIED, 1980). In this study, the malnourished animals presented leucopenia as well as bone marrow and spleen hypoplasia compared to animals of the control group. Animals in the malnourished group which had received LPS also presented marrow hypoplasia and peripheral leucopenia compared to the control animals. The apparent difficulty in leucocyte response to bacterial infections in PEM is, according to Suda et al. (1976) is due to a reduction in the reserve compartment of the bone marrow and changes in the extracellular matrix (XAVIER et al., 2007) and not due to the primary impairment of leucocyte mobilization from blood to tissue (CARLOS; HARLAN, 1994).

The primary humoral immune response requires several days to occur and therefore in the early stages of host defense antibody-independent mechanisms of complement activation are extremely important (BOHLSON et al., 2001). The role of the complement system as a system merging early-phase innate immunity with later-phase acquired immunity has been extensively established in literature. C3 is a key protein of the complement system. It can be activated by four pathways: (1) the classical or alternative pathway, (2) the mannan binding protein pathway, (3) the C-reactive protein pathway, and (4) the natural IgM pathway of innate immunity (SAKAMOTO; FUJISAWA; NISHIOKA, 1998).

The effects of deficiencies in one of these aforementioned pathways, particularly in the alternative pathway, increase the host's susceptibility to infection. The biological effects of the complement system include the promotion of chemotaxis and anaphylaxis, opsonization, and phagocytosis of microorganisms. C3 is the most important central molecule of the complement system because both the classic and the alternative pathways activate it, and its activation products mediate opsonization and anaphylactic activity and also activate the terminal pathway (MICHAEL; FRANK, 2000; MOLINA, 2004).

C3 activation results in a variety of immune reactions such as immune adherence, phagocytosis, antibody response, cytolysis, inflammation, and killing of pathogenic microorganisms (SAKAMOTO; FUJISAWA; NISHIOKA, 1998). In humans and in experimental animals, reduced levels of complement, especially of C3, were observed in hosts whose defense against infection was deficient (CARVALHO; MARTINS; CARNEIRO, 1984; SAKAMOTO; FUJISAWA; NISHIOKA, 1998).

It has been established now that nutritional deficiency is commonly associated with impaired immune responses, particularly cell-mediated immunity, phagocyte function, cytokine production, secretory antibody response, antibody affinity and the complement system (CHANDRA, 1999). Complement is an essential opsonin and the concentrations and activity of most complement components are decreased. The best documented is a reduction in C3, CS, factor B, and total hemolytic activity. There is a slight reduction in opsonic activity of plasma. Furthermore, metabolic activation and intracellular destruction of bacteria are also reduced (CHANDRA, 1997).

In our study, the C3 component and total complement were determined in control and malnourished animals without stimulus or stimulated with LPS. Our results showed lower concentrations of C3 in malnourished animals in both cases. An interesting result was observed after the LPS stimulus, when the control animals showed an increase in the values of CH50 and C3, thus indicating an increase of the non-specific immunological response. In our model of malnutrition, we observed less production of IL-1, IL-6 and TNF-α after the LPS stimulus (FOCK et al., 2007). In the present work the lower serum concentration of C3 Complement in malnourished mice stimulated with LPS can be a consequence of the lower capacity of synthesis of pro-inflammatory substances such as IL-1, IL-6 and TNF-α. These cytokines induce liver proteins of acute phase production such as C3 component of the system Complement (KOJ et al., 1988). Moreover, the reduction of protein ingestion and, consequently essential amino acids in the diet, as branched-chain amino acids, favored the lower serum C3 concentration in malnourished animals, since branched-chain amino acids are involved in the liver turnover (JIMÉNEZ et al., 1991; HOLECEK; SPRONGL; TILSER, 2001; MAY; BUSE, 1989).

The experimental data lead us to infer that the immune deficiency response is not only due to decreased C3 complement levels. The principal cause is the inability to produce more C3 in response an infection.

CONCLUSION

Our data show that animals in an inflammatory model of PEM present a decrease in all parameters evaluated and do not present an immune response in the same intensity or manner as well nourished animals.

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