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Evaluation of Possible Failure of the Mononuclear Phagocyte System after Total Splenectomy in Rats

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ABSTRACT

Young and adult Wistar rats were submitted to total splenectomy and compared to animals not submitted to any surgical manipulation in order to evaluate the phagocytic function of spleen. The animals were infected with Escherichia coli labeled with technetium-99m and killed 20 minutes later. Liver, lung, spleen and a blood clot sample were taken. No significant differences were found in the percentage of bacterial radioactivity uptake in mononuclear phagocyte system (MPS) organs in young and adult splenectomized rats. However, phagocytosis index by macrophages of MPS organs was smaller in splenectomized animals than in control group. Splenectomized rats were associated with a higher blood bacterial radioactivity uptake than animals of the control group (p<0.0001) due to a larger bacterial remnant in the bloodstream. This finding suggested that some failure in the MPS occurred in the absence of the spleen, demonstrating the need to develop alternative surgical techniques for total splenectomy.

Key words: Spleen, Splenectomy, Technetium-99m, *Escherichia coli*, Phagocytosis, Mononuclear phagocyte system

INTRODUCTION

Being the major lymphoid organ of the human body, the spleen performs critical immunological functions such as bacterial clearance from the bloodstream and early antibody production against various antigenic particles. These functions account for the special role the spleen plays in host defense during bacteremia (Altamura et al., 2001). The organ primarily produces monocytes and lymphocytes and participates in the phagocytosis of foreign particles, bacteria, viruses and leukocytes, besides producing serum factors such as opsonins that strongly stimulate phagocytosis (Altamura et al., 2001; Saba, 1970; Timens and Leemans, 1992). In mammals, the main opsonins are the third fraction of complement, C_3 (C_{3a} and C_{3b}) and the F_c portion of immunoglobulin G, with other components like fibronectin and protein-C reactive being also present (Clayer et al., 1994; Hosea et al., 1981; Pachter and Grau, 2000; Saba, 1970; Sumaraju et al., 2001). Two other substances related to macrophage activation,

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tuftsin and properdin are also produced in the spleen (Chu et al., 1984).

Besides performing these activities, the spleen is also a voluminous filter through which 4% of the blood volume circulates per minute, with the removal of altered and senescent erythrocytes, and corpuscle inclusions such as Howell-Jolly, Heinz and Pappenheimer bodies (Clayer et al., 1994; Sumaraju et al., 2001). In 1969, Diamond called attention to what he named overwhelming postsplenectomy infection (OPSI), a clinical entity distinct from other types of sepsis and bacteremias occurring in individuals with a preserved spleen, alerting to the risk of the aspleny. Singer, in 1973, published a detailed study showing that the risk of OPSI development was higher than previously expected. The author emphasized that sepsis could occur at any time after surgery, both in adults and children, and that its risk was independent of any surgical indication for splenectomy. Several experimental studies have shown that aspleny is related to reduction of bacterial clearance from the bloodstream. This work was aimed at evaluating the distribution of *Escherichia coli* labeled with technetium-99m in the main organs of mononuclear phagocyte system (MPS) in Wistar rats and determining the remaining bacteria in the bloodstream of normal and splenectomized animals.

MATERIAL AND METHODS

Fifty-eight Wistar rats of both sexes were divided in two groups: I - control, not submitted to any surgical manipulation; and II - submitted to total splenectomy. Each of these groups was divided in two subgroups: A - twenty nine young rats, weighing 100 to 150 g (group I - 5 male and 9 females; group II - 8 males and 7 females); and B - twenty nine adult rats, weighing 250 to 300 g (group I - 4 males and 10 females; group II - 6 males and 9 females). The animals were placed in appropriate cages, five to a cage at most, and received rat chow and water *ad libitum*.

After a 6-hour fast, the animals were submitted to inhalation anesthesia with halothane, abdominal trichotomy, disinfection with iodopividine and placement of surgical fields. Total splenectomy was performed by supraumbilical midline laparotomy. The abdomen was carefully searched for the presence of any accessory splenic tissue. Laparorrhaphy was carried out in two planes

(peritoneal-aponeurotic plane and skin), using 3-0 polyglycolic acid sutures. After recuperation of physical activity, the rats were replaced into their cages without alimentary restriction.

Escherichia coli (strain AB1157) was labeled with technetium-99m (Tc-99m) in the form of sodium pertechnetate (TcO_4Na) as described previously. (Bernardo-Filho et al., 1986; Diniz et al., 1999) Briefly, a sample of *E. coli* culture was added to Luria and Burrows (LB) medium and incubated for 15 to 18 hours overnight at 37ºC with shaking. A 200-µl aliquot was then removed, placed in LB and incubated for an additional two hours with shaking at the same temperature (replating). A 2-ml aliquot was removed from the replated sample, homogenized and centrifuged at 4000 rpm for 25 minutes (clinical centrifuge) at room temperature. After successive washes with NaCl solution, a LB-free suspension was obtained. This suspension was placed in a vacuum tube and stannous chloride ($SnCl_2.2H_2O$) was added at a concentration of 30 µg/ml and incubation for 15 minutes at 37ºC with shaking was performed. Then, 0.5 ml of Tc-99m at an activity of 1.85 MBq/ml was added and the suspension homogenized, incubated for 10 minutes at 37ºC with shaking and centrifuged at 4000 rpm for 25 minutes. The supernatant and the precipitate (pellet) resuspended in 1.0 ml 0.9% NaCl were placed in separate tubes for radioactive counting with a gamma scintillation counter for the determination of the percentage of labeled bacteria that was higher than 95%. After counting, 0.9% NaCl was added to the tube containing the bacterial pellet at a volume sufficient to allow the experiment described below; the suspension was then homogenized until being adequate for inoculation. Before use, two aliquots of the suspension were removed, titrated, placed on Petri dishes containing LB-gel (1.5% agar-agar) as culture medium and randomly spread with glass beads. The aliquots were titrated with the utilization of 50 µl of the bacteria suspension. This suspension was added to an assay tube containing 5.0 ml of 0.9% NaCl. From this tube, 50 µl were taken out and put in a second assay tube, also containing 5.0 ml of 0.9% NaCl. Following this, 100 µl were taken out from this second tube and put in a third one containing 0.9 ml of 0.9% NaCl. This final volume of 1.0 ml was put in Petri dishes and placed in an incubator for 24 hours at 37ºC for the determination of the number of colony-

forming units (CFU). A bacterial concentration of 10^8 CFU was thus obtained.

Sixteen weeks after the beginning of the experiment, the animals were submitted to inhalation anesthesia with halothane, cervical and abdominal trichotomy and disinfection with iodopovidine. Rats were inoculated with Tc-99m-labeled *E. coli* through direct transverse cervicotomy by dissecting the internal jugular vein. Twenty minutes after inoculation, the animals were killed with a halothane overdose and submitted to midline thoracolaparotomy. The caudal vena cava was cut, leading to intra-abdominal bleeding which in turn resulted in the formation of a blood clot. The liver, lung and spleen, as well as the blood clot, were removed, weighed and placed in appropriate tubes for radioactive counting with a gamma scintillation counter.

A standard dose containing the same volume and the same activity of the Tc-99m-labeled *E. coli* suspension inoculated into the animals was used for calculation. The standard count was considered to be 100% of the radioactivity inoculated into the animals. Percent radioactivity uptake was calculated for each sample using the formula:

$$\% \text{ Uptake} = \frac{\text{Sample count per minute (cpm)}}{\text{Standard cpm}} \times 100$$

Taking into consideration the mass of the liver, lung, spleen and blood clot, percent radioactivity uptake per g tissue was calculated using the formula:

$$\% \text{ Uptake} / g = \frac{\% \text{ Tissue uptake}}{\text{Sample (g)}}$$

Percent radioactivity uptake by each organ (total mass) was calculated using the formula:

$$\% \text{ organ uptake} = \% \text{ uptake/g} \times \text{total organ mass(g)}$$

Blood (1.0 ml) was collected from the caudal vena cava during the laparotomies for organ removal from ten young and ten adult rats, placed in assay tubes and left to stand for 24 hours. The tubes were centrifuged to completely separate serum and blood clots. The clots were weighed, with each milliliter of blood resulting on average in a 0.49 g clot in both subgroups. A rat weighing 250 g contains 16 ml of blood and this corresponds to approximately 7.85 g of clot material (Diehl et al., 2001). Based on each blood clot mass the percent bacterial radioactivity uptake in the whole blood was estimated for each animal.

The distribution of captured bacteria was normalized, considering that radioactivity uptake only by liver, lung and spleen, plus blood (bacteria remaining in the bloodstream) was 100%. For statistical analysis, radioactivity uptake of Tc-99m-labeled bacteria by the liver, lung and spleen, as well as of the bacteria remaining in the bloodstream, was compared between groups and subgroups. The Student t-test was applied to pairs of groups and subgroups of animals. The level of significance was set at 5%.

RESULTS

An analysis of the percentage of bacterial radioactivity uptake by MPS organs as well as by bacteria remaining in the bloodstream is shown in Table 1.

Table 1 - Percent bacterial radioactivity uptake, in young and adult animals of control and splenectomized groups.

Groups		Young animals				Adult animals			
		Liver	Lung	Spleen	Blood	Liver	Lung	Spleen	Blood
I	Mean	77.2551	17.6436	2.5126	2.5887	78.4926	16.4909	2.2901	2.7264
	SD	4.6719	3.8343	0.7999	1.5226	3.8810	4.2204	0.8773	0.9827
II	Mean	77,3743	18.3786	- - -	4.2471	75.9407	19.0428	- - -	5.0165
	SD	5.3058	4.6277	- - -	2.1774	3.2519	3.4521	- - -	1.7609

SD - Standard deviation; I - Control group; II - Total splenectomy

Comparative analysis between young and adult animals within each group showed no difference in percent bacteria radioactivity uptake in MPS

organs. Both young and adult splenectomized rats presented a higher radioactivity uptake of bacteria remaining in the bloodstream (p=0.0257 and p=0.0002, respectively) than control animals. Comparative analysis between groups even when all animals of each group were considered without age distinction showed a higher percent radioactivity uptake of bacteria in the bloodstream of splenectomized rats than in controls (Table 2).

Table 2 - Comparative analysis in percent bacterial radioactivity uptake in MPS organs between young and adult animals (young - A *vs*. adults - B), in a same group and between the two groups (I-II), with and without age distinction, by Student t-test (p values).

Subgroups	Liver	Lung	Spleen	Blood
IA – IB	0.4527	0.4562	0.4895	0.7783
IIA – IIB	0.3799	0.6593	- - -	0.2964
IA – IIA	0.9494	0.6465	- - -	0.0257*
IB – IIB	0.0649	0.0851	- - -	0.0002*
I – II (without age distinction)	0.2892	0.1248	- - -	0.000*

* Significant difference; I - Control group; II - Total splenectomy

DISCUSSION

The importance of the spleen in the removal of encapsulated bacteria from the bloodstream (*Streptococcus pneumoniae* - 50% to 90% of all infections and 60% from fatal cases of OPSI -, *Haemophilus influenzae* type B and *Neisseria meningitidis*) has been intensively studied and is now universally accepted (Altamura et al., 2001; Bohnsack and Brown, 1986; Diamond, 1969; Leemans et al., 1999; Moxon et al., 1980; Singer, 1973; Sumaraju et al., 2001). This immunoprotective effect has been tested in several experimental studies by bacterial challenging, especially in studies on mortality and clearance capacity. These investigations involve a wide diversity of models in terms of animal species and age, bacterial species, inoculation route, time elapsed since total splenectomy, as well as evaluation methods.

The study of bacterial phagocytosis by MPS organs constitutes an experimental model apparently adequate to verify the efficacy of bacterial clearance from the bloodstream in splenectomized animals. It is believed that bacterial clearance represents a more complex method than colloidal substance clearance, providing safer indications of the phagocytic activity of MPS organs (Brown et al., 1981; Hansen and Singer, 2001; Scher et al., 1982).

We used an *E. coli* strain AB1157 due to the wide knowledge available about it. Also it is part of the normal human intestinal flora and responsible for about 12% of overwhelming postsplenectomy infection. These Gram-negative bacteria predominate in infections found in elderly splenectomized patients whose health is affected by chronic diseases (Hansen and Singer, 2001; Singer, 1973). For the study of the response to inoculation with Gram-negative bacteria, the intravenous route seems to mimic better the clinical situation, since the sepsis caused by these bacteria originates from the digestive and not from the respiratory system.

Several radionuclides - carbon-14, gallium-67, hydrogen-3, rhenium-188, technetium-99m, and others - have been used to label different biological structures. The physical characteristics of Tc-99m favor its preference for biological purposes since involves a simple labeling method, which, however, is a precise, effective, rapid, easily available and of low cost (Bernardo-Filho et al., 1986; Diniz et al., 1999). One of the many applications of Tc-99m is the possibility of determining the biological behavior of experimental animals in response to bacterial inoculation. Bacteria labeled with this technique forms a bacterium-Tc-99m complex which is stable for more than 24 hours, indicating strong binding of Tc-99m atoms with bacterial cell components and producing a rate of labeled bacteria almost always higher than 95%, favoring the reliability to the experiment (Bernardo-Filho et al., 1986; Diniz et al., 1999).

The literature shows that more than 90% of the bacteria are removed from the bloodstream in the first five minutes after their inoculation (Holdsworth et al., 1989; Scher et al., 1982). Nevertheless, in our experiments we waited 20 minutes after intravenous inoculation before

killing the animals in order to allow the phagocytosis of the highest possible number of bacteria by the macrophages of MPS organs. Spleen, liver and lung account for more than 95% of the macrophages from MPS organs (Moxon et al., 1980; Saba, 1970; Scher et al., 1982). Since the determination of bacteria in the other MPS organs - Peyer patches, bone marrow and lymph nodes – has not been routinely feasible, we normalized the distribution of bacterial radioactivity uptake considering that uptake in the main MPS organs - liver, lung and spleen -, plus the uptake of bacteria remaining in the bloodstream, was 100%.

The liver is the largest MPS organ and, as expected, presented the highest bacterial radioactivity uptake. No significant differences in bacterial phagocytosis were detected between young and adult animals within the same group. The evidence that aspleny is related to slower bacteria blood clearance is confirmed by the occurrence of a higher radioactivity uptake of bacteria remaining in the bloodstream in splenectomized animals compared to control regardless age and sex.

According to the literature, the spleen is responsible for 25 to 30% of MPS-mediated blood clearance (Malangoni et al., 1985; Patel et al., 1982; Petroianu et al., 1992; Saba, 1970; Scher et al., 1982). These studies, which have been mainly conducted using colloidal substances, disagree with the results of the present study obtained with bacteria. The participation of MPS organs in the phagocytosis of bacteria differed from that observed with colloidal substances. While the spleen shows the second highest phagocytic index for colloidal substances, followed by the lung (about 5% to 10% of the phagocytic activity), with bacteria this ratio is inverted (Bohnsack and Brown, 1986; Holdsworth et al., 1989; Hosea et al., 1981). In both situations the liver, perhaps due to its larger mass, is the organ with the greatest participation in blood clearance. The reasons for this different behavior in the phagocytosis of colloidal and bacterial substances are unknown. It is possible that due to its important phagocytic action in bacterial clearance the lung is the organ more compromised by infectious complications in splenectomized patients. This finding perhaps could contribute to elucidate the mechanism by which pneumonia is the most common cause of sepsis in asplenic patients.

Silva (2000) showed increased bacterial radioactivity uptake in the lungs of adult rats intravenously inoculated with *E. coli* after partial splenectomy. Shennib et al. (1983) observed a transitory suppression of pneumococcus phagocytosis by lung macrophages in young splenectomized rats. However, when splenic fragments were left in the abdominal cavity this did not occur. These results suggest that, with a smaller functioning splenic mass after partial or subtotal splenectomy, the alveolar macrophages suffer alterations in their phagocytic activity in an attempt to compensate for an eventual decrease of splenic phagocytic index.

Our finding suggested that some failure in the MPS occurred in the absence of the spleen, demonstrating the need to develop alternative surgical techniques to total splenectomy in patients requiring spleen removal.

RESUMO

Ratos *Wistar* jovens e adultos foram submetidos a esplenectomia total e comparados a animais não submetidos a qualquer procedimento cirúrgico para avaliar essa função fagocitária do baço. Dezesseis semanas após, os animais foram inoculados, por via intravenosa, com uma suspensão de *Escherichia coli* marcada com tecnécio-99m e, vinte minutos após, foram mortos. Fígado, pulmão, baço e uma amostra de coágulo sangüíneo foram retirados para determinação da captação bacteriana. Não foram encontradas diferenças significativas no percentual de captação bacteriana nos órgãos do SMF entre ratos esplenectomizados jovens ou adultos. Entretanto, o índice fagocitário dos macrófagos dos órgãos do Sistema Mononuclear Fagocitário (SMF) foi menor nos animais esplenectomizados que no grupo controle. Ratos esplenectomizados apresentaram um percentual de captação de bactérias na corrente sangüínea maior que os animais do grupo controle (p<0,0001), devido a um maior remanescente de bactérias na corrente sangüínea. Esse achado sugere que, na asplenia, ocorre alguma falha no SMF, demonstrando a necessidade do desenvolvimento de técnicas operatórias alternativas à esplenectomia total para pacientes que necessitam da remoção do baço.

REFERENCES

Altamura, M.; Caradonna, L.; Amati, L.; Pellegrino, N. M.; Urgesi, G. and Miniello, S. (2001), Splenectomy and sepsis: The role of the spleen in the immune-mediated bacterial clearance. *Immunopharmacol. Immunotoxicol.*, **23**, 153-161.

Bernardo-Filho, M.; Pereira, J. A. A. and Boasquevisque, E. M. (1986), Labeling of *Klebsiella pneumoniae* with technetium-99m - A preliminary communication. *Rev. Microbiol.*, **17**, 188-193.

Bohnsack, J. F.; Brown, E. J. (1986), The role of the spleen in resistance to infection. *Annu. Rev. Med.*, **37**, 49-59.

Brown, E. J.; Hosea, S. W. and Frank, M. M. (1981), The role of complement in the localization of *pneumococci* in the splanchnic reticuloendothelial system during experimental bacteremia. *J. Immunol.*, **126**, 2230-2235.

Chu, D. Z.; Nishioka, K.; El-Hagin, T.; Hopfer, R. L. and Romsdahl, M. M. (1984), Effects of tuftsin on postsplenectomy sepsis. *Surgery*, **97**, 701-706.

Clayer, M. T. R.; Drew, P. A.; Leong, A. and Jamieson, G. G. (1994), IgG-mediated phagocytosis in regenerated splenic tissue. *Clin. Exp. Immunol.*, **97**, 242-247.

Diamond, L. K. (1969), Splenectomy in childhood and the hazard of overwhelming infection. *Pediatrics*, **43**, 886-889.

Diehl, K. H.; Hull, R.; Morton, D.; Pfister, R.; Rabemampianina, Y.; Smith, D.; Vidal, JM and van de Vorstenbosch Cor. (2001), A good practice guide to the administration of substances and removal of blood, including routes and volumes. *J. Appl. Toxicol.*, **21**, 15-23.

Diniz, S. O. F.; Resende, B. M.; Nunan, E. A.; Simal, C. J. R. and Cardoso, V. N. (1999), ^{99m}Technetium labelled *Escherichia coli. Appl. Radiat. Isot.*, **51**, 33-36.

Hansen, K. and Singer, D. B. (2001), Asplenic-hyposplenic overwhelming sepsis: Postsplenectomy sepsis revisited. *Pediatr. Devel. Pathol.*, **4**, 105-121.

Holdsworth, R. J.; Neill, G. D.; Irving, A. D. and Cuschieri, A. (1989), Blood clearance and tissue distribution of ^{99}Tc-labelled pneumococci following splenectomy in rabbits. *Br. J. Exp. Path.*, **70**, 669-677.

Hosea, S. W.; Brown, E. J.; Hamburger, M. I. and Frank, M. M. (1981), Opsonic requirements for intracellular clearance after splenectomy. *N. Engl. J. Med.*, **304**, 245-250.

Leemans, R.; Manson, W.; Snijder, J. A. M.; Smith, J. W.; Klasen, H. J.; Hauw-The, T. and Timens W. (1999), Immune response capacity after human splenic autotransplantation. *Ann. Surg.*, **229**, 279-285.

Malangoni, M. A.; Dawes, L. G.; Droege, E. A.; Rao, A. S.; Collier, D. and Almagro, U. A. (1985), Splenic phagocytic function after partial splenectomy and splenic autotransplantation. *Arch. Surg.*, **120**, 275-278.

Moxon, E. R.; Goldthorn, J. F. and Schwartz, A. D. (1980), *Haemophilus influenzae* b infection in rats: Effect of splenectomy on bloodstream and meningeal invasion after intravenous and intranasal inoculations. *Infect. Immunol.*, **27**, 872-875.

Pachter, H. L.; Grau, J. (2000), The current status of splenic preservation. *Adv. Surg.*, **34**, 137-174.

Patel, J. M.; Williams, J. S.; Naim, J. O. and Hinshaw, J. R. (1982), Protection against pneumococcal sepsis in splenectomized rats by implantation of splenic tissue into an omental pouch. *Surgery*, **91**, 638-641.

Petroianu, A.; Simal, C. J. R. and Barbosa, A. J. A. (1992), Impairment of phagocytosis by mammalian splenic macrophages by 99Tc^m sulphur colloid. *Med. Sci. Res.*, **20**, 847-849

Saba, T. M. (1970), Physiology and physiopathology of the reticuloendothelial system. *Arch. Intern. Med.*, **126**, 1031-1052.

Scher, K. S.; Wroczynski, F. and Coil, J. A. (1982), The effect of splenectomy on gram-negative bacteremia. *J. Trauma*, **22**, 407-409.

Shennib, H.; Chiu, R. C. J. and Mulder, D. S. (1983), The effects of splenectomy and splenic implantation on alveolar macrophage function. *J. Trauma*, **23**, 7-12.

Silva, R. G. (2000), Fagocitose da *Escherichia coli* pelo sistema mononuclear fagocitário, após esplenectomias subtotal, parcial e total, em rato. Ph.D. Thesis, Minas Gerais Federal University, Belo Horizonte, Brazil.

Singer, D. (1973), Postsplenectomy sepsis. *Perspect. Pediatr. Pathol.*, **1**, 285-311.

Sumaraju, V.; Smith, L. G. and Smith, S. M. (2001), Infectious complications in asplenic hosts. *Infect. Dis. Clin. North Am.*, **15**, 551-565.

Timens, W. and Leemans, R. (1992), Splenic autotransplantation and the immune system. Adequate testing required for evaluation of effect. *Ann. Surg.*, **215**, 256-260.