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APPEARANCE OF IRON-NITROSYL COMPLEXES IN MURINE L5178Y LYMPHOMA AS A FUNCTION OF THE FORM OF GROWTH AND THE TYPE OF HOST

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Murine L5178Y lymphoma growing in the form of intraperitoneal ascites, both in natural, syngeneic DBA/2, and in allogeneic Swiss hosts, is much more aggressive than solid tumors. The main aim of the present paper was to find out whether this difference can be correlated with the level and quality of paramagnetic complexes of iron II and nitric oxide (NO), which can be easily detected by EPR at X band and 77 K in both types of the materials. We found that the main type of Fe-NO complexes detectable in ascites is iron-heme nitrosyl complex of NO and hemoglobin. The signal of non-heme, dinitrosyl iron complexes (DNIC) can be observed only in white ascites revealing low substitution of erythrocytes (less than 1×10^8 cells/ml). In Swiss hosts this signal appeared spontaneously in ca. 50% of white ascites, whereas in DBA/2 mice only the huge excess of exogenous NO (40 mM) induced this signal. DNIC signals are observed almost in every solid tumors growing in Swiss host, whereas in DBA/2 hosts ca. 40% of solid tumors are devoid of this signal, and in general, the intensities of all types of Fe-NO complex signals are lower in DBA/2 than in Swiss hosts. As distribution of blood hemoglobin in ascitic fluid is homogenous, while vascularization of solid tumor tissue is variable, we conclude that not only the host-dependent level of NO, but also the amount and distribution of blood in the L5178Y lymphoma are responsible for the level and quality of Fe-NO complexes detectable by EPR, depending strongly on the form of their growth as ascites or solid tumors.

INTRODUCTION

Murine L5178Y lymphoma was derived from thymic lymphocytes by methylcholantrene in DBA/2 mice in 1952, initially in the ascitic form growing in the peritoneal cavity (Beer, Budzicka, Niepokojczycka, Rosiek, Szumiel & Walicka, 1983). Although this line is mainly used as a model in radiological and oncological investigations *in vitro* (Beer *et al.*, 1983; Kapiszewska, Szumiel & Beer, 1981; Alexander & Mikulski, 1961), there are several reasons for which it has also become an interesting and versatile model to investigate tu-mor-host interactions *in vivo* by EPR spectroscopy:
(1) The dual, ascitic and solid-tumor form of growth *in vivo* (Plonka, Cieszka, Plonka, Pajak, Raczek & Lukiewicz, 1996a; Szczygiel, Pawlus, Plonka, Elas, Szczygiel, Plonka & Lukiewicz, 2004). (2) Growth *in vivo* in several types of hosts, namely DBA/2 (natural, syngeneic inbred host), Swiss (allogeneic outbred host), and C57BL/6 mice (allogeneic inbred host; Plonka *et al.*, 1996a; Plonka, Raczek, Plonka & Lukiewicz, 1996b). (3) An intriguing phenomenon of the so-called "concomittant immunity" against secondary tumors growing in the same organism in parallel to another tumor of this lymphoma (Szczygiel *et al.*, 2004).
(4) Appearance of various kinds and amounts of iron-nitrosyl (Fe-NO) complexes, easy to detect in ascites and solid tumors by EPR spectroscopy at 77 K (Plonka *et al*, 1996a, b; Plonka, Gurbiel & Plonka, 1999a).

Solid tumors of L5178Y lymphoma kill 100% of the DBA/2 hosts, but the mortality of Swiss host is lower (ca. 90%). In the highly immunoincompatible allogeneic inbred host – C57BL/6 mice solid tumors of this lymphoma are also able to initiate growth followed, however, by spontaneous regression in 100% of the hosts (Plonka *et al.* 1996a, 1999a). Due to the ability to accumulate iron (Szumiel, Kapiszewska, Kruszewski, Iwanenko & Lange, 1995) and the lack of other strongly paramagnetic species (e.g. melanin), the level of nitric oxide (NO) produced in the tissues and ascites of this lymphoma and the corresponding amount and type of paramagnetic Fe-NO complexes are expected to depend mainly on the strength of the host anti-tumor defence (Plonka *et al.*, 1999a, 1996a). Their presence can be correlated with the behavior of tumor in particular types of host (Plonka *et al.*, 1999b, 1996a). This makes

L5178Y lymphoma particularly suitable to investigate the role of nitric oxide in tumor growth, the metabolism of iron, and the mechanism of the appearance of iron-nitrosyl complexes in tumors, using EPR spectroscopy.

Although the dynamics of L5178Y lymphoma growth in various hosts is different, the particularly striking difference can be observed in regard to the aggressiveness of ascitic and solid tumor forms. Ascites kills the DBA/2 and Swiss hosts very rapidly, as compared to the solid tumors. Interestingly, the solid-tumor phenotype of the *in vivo* growing lymphoma can be reversed -in ca. 15-20% animals (both types) pre-inoculated with solid tumors, spontaneous ascites develops, which kills the hosts twice as quickly as the solid tumor (Szczygiel *et al.*, 2004). Moreover, while L5178Y lymphoma in the form of solid tumors never kills the allogeneic C57BL/6 hosts and always undergoes spontaneous regression (Plonka *et al.*, 1999a), it sometimes grows as an ascites in this host revealing similar agresiveness as in other types of hosts (Kuter, 2000).

We have already shown that nitrosyl hemoglobin (HbNO), and dinitrosyl non-heme iron (DNIC) complexes are detectable in the lymphoma according to the degree of immunoincompatibility between the tumors and their hosts (Plonka *et al.*, 1999a, 1996a). The DNIC-type signals are detectable mainly in the situation of a strong antitumor defence (*e. g.* in Swiss hosts; Plonka *et al.*, 1999a, 1996a, b) but the detection of HbNO complexes is dependent also on the degree of tissue vascularization (Plonka *et al.*, 1996a). In quest of other factors which may determine the appearance and the intensity of various Fe-NO complexes, we employ here the *in vivo* model of L5178Y lymphoma to show that the differences in the level and quality of Fe-NO complexes are also a result of the ascitic, or solid tumor form, and to what degree it can be modulated by the kind of the host -DBA/2 or Swiss mice.

MATERIALS AND METHODS

Chemicals, reagents and media

Phosphate buffered saline (PBS) was obtained from BIOMED Wytwornia Surowic i Szczepionek, Lublin, Poland; sodium nitrite ($NaNO_2$) from Zaklady Azotowe Chorzow, Poland; sodium dithionite ($Na_2S_2O_4$), from Sigma-Aldrich, St. Louis, MO, USA. In *in vitro* model experiments all the reagents were dissolved in PBS bubbled for about 20 min with nitrogen in order to remove oxygen from the solutions.

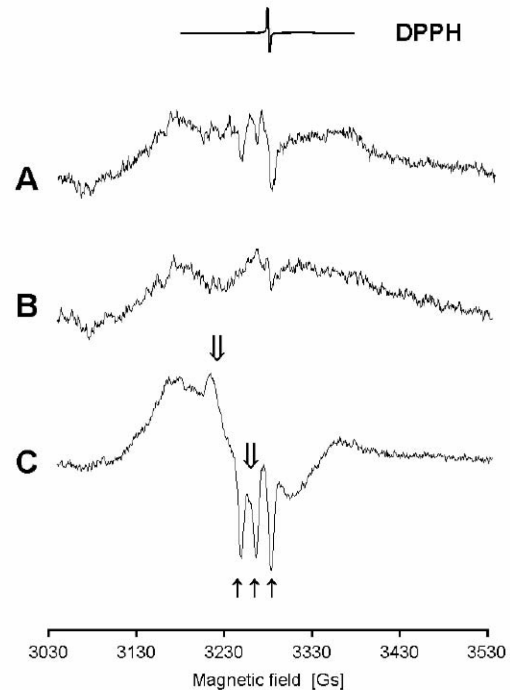

Fig. 1. Representative EPR spectra of ascites with blood (**A**), ascites almost devoid of blood (**B**), and a solid tumor (**C**) of L5178Y lymphoma growing in the syngeneic DBA/2 hosts. Arrows indicate the hyperfine structure of the HbNO signal, wide arrows – the low field and high-field features of the anisotropic DNIC signal. Instrumental gain: 4×10^5 (A, B, C), other parameters of the EPR assay – see Material and Methods, DPPH -the free radical standard signal (a tiny crystal of 1,1-diphenyl-2-picrylhydrazyl).

Animals and tumors

In all the experiments the basic version of murine lymphoma L5178Y (*i. e.* L5178Y-R) growing *in vivo* in the form of s.c. solid tumors or i.p. ascites was used. The line has been maintained in our laboratory for more than 10 years. Inbred mice DBA/2 were the natural, syngeneic host for this tumor line and outbred mice Swiss served as a specific, allogeneic host. Mice (Animal Breeding Facility of the Jagiellonian University Collegium Medicum, Institute of Pediatrics, Krakow, Poland) were 8-10 weeks old (male and female). They were fed with standard rodent chow and water *ad libitum*. Solid tumors were inoculated s.c. using 2×10^7 ascitic cells or 30 mg of tiny solid tumor pieces. To induce ascites 5×10^6 of cells were inoculated i.p. On day 8-12 after the inoculation animals were euthanized and tumors or ascites extracted for EPR measurement. The particular attention was paid to the color of the ascitic

fluids, which ranged from white to intensively red, and to the number of erythrocytes in the ascitic fluid. In parallel, the total volume of blood extracted from the left ventricle after euthanasia was measured. All the experiments were approved by the Local Bioethical Commission in Krakow (opinion 15/OP/2002 and 18/OP/2003).

Experiments with exogenous NO

The solution of nitric oxide was obtained by reduction of sodium nitrite using the 2.5-fold excess of sodium dithionite (Wennmalm, Lanne & Petersson, 1990). The solution (0.2-0.3 ml) was added in bolus directly to the peritoneal cavity of a host directly after euthanasia, or the solution was mixed with ascites in a test tube *ex vivo* with ascitic fluid or blood (0.1 ml/0.2 ml), so as to obtain the final concentrations of NO 80 M or 40 mM in the samples. Pilot trials confirmed that the procedure of mixing *ex vivo* or in the peritoneal cavity did not significantly affect the EPR signal of the ascites.

EPR measurements and statistical analysis of the data

The solutions, ascites or pieces of tumors were placed in glass tubes (inner diameter 4.7 mm) and immediately frozen in liquid nitrogen. The obtained icicles were then pushed into a quartz Dewar flask and measured at 77 K in liquid nitrogen. EPR measurements were performed using a Varian E-3 spectrometer with a rectangular TE 102 cavity. EPR spectra were recorded at X-band (9.15 GHz), 10 Gs modulation amplitude, 3280 ± 250 Gs field center and sweep, 4 mW microwave power, 0.3 s time constant, 240 s acquisition time, and 1×10^3-4×10^5 receiver gain. The spectra were recorded in a digital form (the average of 3 records was used as a working spectrum). In all cases the EPR signal amplitudes were normalized according to the constant mass of a sample – 400 mg on the base of a calibration curve prepared for the particular resonant cavity and the applied sample geometry. The intensity of DNIC signals was expressed as the amplitude of the signal at $g_\perp$=2.035 (Plonka *et al.*, 1999a; Vanin & Kleschyov, 1998, Bastian, Yim, Hibbs & Samlowski, 1994), and of HbNO – as the amplitude of the first constituent of the hyperfine splitting (see Szczygiel *et al.*, 2004). All the quantitative data were expressed as means ± SEM. The statistical significance of the observed differences was tested with the independent, two tailed Student *t*-test.

RESULTS AND DISCUSSION

Blood content in ascites and in peripheral circulation of the hosts

One of the main symptoms of killing the hosts by ascites was blood extravasation to the peritoneal cavity, which was manifested by the transition of the default white color of the ascites to pink and red. The content of red blood cells in ascites tended to increase in parallel to this transition, from about 1-2×10^8 cells/ml for the ascites with a hardly noticeable pink shade, to ca. 2×10^9 cells/ml for intensively red ascites. At the same time, the mean volume of the peripheral blood removed from the left ventricle dropped from 1.05 ± 0.06 ml (N = 6) for mice without ascites and 0.93±0.04 (N = 3) for mice with white ascites to 0.53±0.08 ml (N = 6) for mice with red ascites ($0.01>p\geq 0.001$; Swiss). Low amount of blood in the animals with intensively red ascites may indicate drastic extravasation of blood to the peritoneal cavity. As these animals revealed also other symptoms of terminal stages of the disease (cachexia, apathy),

Table 1. Correlation between the amount of blood and the type of murine host with the appearance (%) and the intensity (mean amplitudes±SEM, [a.u.]) of EPR signals of various Fe-NO complexes detected in L5178Y lymphoma. Data was collected from 141 DBA/2 (70 ascites and 71 tumors) and 106 SWISS mice (64 ascites, 42 tumors). All the differences between corresponding white and red ascites, and corresponding materials from DBA/2, and Swiss mice are statistically important ($0.05>p\geq 0.01$; *** $p<0.001$). White ascites in contrast to red ascites contained less than 1×10^8 erythrocytes/ml. Parameters of the EPR assay – see Material and Methods.

Ascites							Tumors			
Mice	Type of material	% ascites	HbNO signals		DNIC signals		HbNO signals		DNIC signals	
			%	Intensity	%	Intensity	%	Intensity	%	Intensity
DBA/2	White	11	0	0	0	0	91	72 ±8***	66	109 ±13
	Red	89	66	10 ±2	0	0				
Swiss	White	13	88	92 ±28	50	123 ±21	100	166 ±22***	97	164 ±20
	Red	87	80	47 ±8	0	0				

the blood content in the ascites seemed to be correlated with the progression of the lymphoma.

To illustrate the dependence of the types and intensity of EPR signals on the blood content in the ascites, it was convenient to divide the test groups in an easily reproductable manner into two separate sub-sets of ascites with high and low content of blood, and to contrast them. As the ascites changed visually from white to pink and red while the number of erythrocytes exceeded 1×10^8, we use this limit to attribute an ascites to the group of high or low blood content.

Appearance of Fe-NO complexes in L5178Y lymphoma growing in DBA/2 hosts

In the syngeneic DBA/2 hosts, the only type of Fe-NO complexes detectable in the ascites of L5178Y lymphoma was HbNO (g = 2.012, A^N= 1.7 mT; Ascenzi, Coletta, Desideri, Polizio, Condo & Giardina, 1990; Kon, 1968), which was limited only to the materials naturally containing high level of blood (Fig. 1A). In white ascites, which did not contain the excess of blood, the HbNO signal was hardly ever detectable (Fig. 1 B). This observation can be explained by the fact that DBA/2 is the natural (syngeneic) host for L5178Y lymphoma, and does not develop any strong defense against it. Therefore, the level of NO, and the accompanying level of endogenous Fe-NO complexes is usually low (Plonka *et al.*, 1999a, 1996a; Bastian *et al.*, 1994) It was indicated not only by low intensity or total lack of their EPR signals, but also by the predominance of HbNO-type signals, mainly of the 5-coordinate-complex character (Plonka et al., 1999a; Hille, Olson & Palmer, 1979). The question, however, remains why non-heme iron complexes (DNIC; $g_\perp$= 2.04, g_{II} = 2.014; Vanin & Kleschyov, 1998; Drapier, Pellat & Henry, 1991; Vanin, Blumenfeld & Chetverikov, 1967) were never detected in the ascitic fluid of DBA/2 mice despite their appearance in ca. 60% of solid tumors (Fig. 1C, Tab.1)?

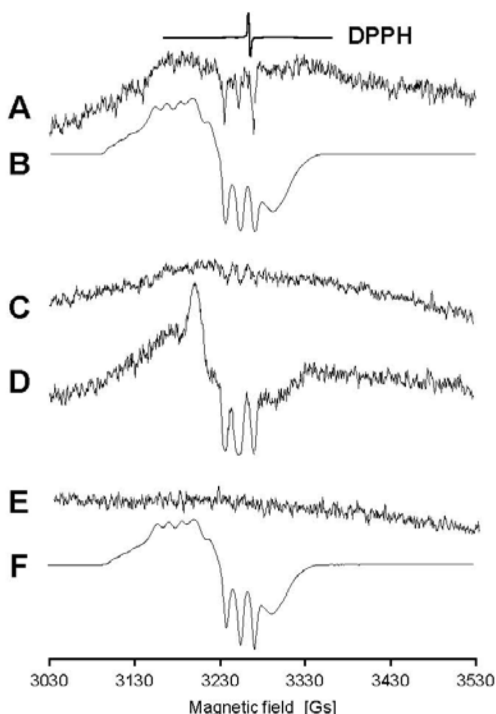

Fig. 2. EPR spectra of L5178Y lymphoma ascites growing in the peritoneal cavity of the syngeneic DBA/2 host with the addition of exogenous nitric oxide: red ascites with endogenous blood and reduced 80 µM and 40 mM sodium nitrite (respectively: **A, B**), white ascites almost devoid of blood, supplemented with reduced 80 µM and 40 mM sodium nitrite (respectively: **C, D**), control peripheral blood (**E**) with reduced 40 mM sodium nitrite (**F**). Instrumental gain: 4×10^5 (A, C, D, E), 2×10^3 (B) and 1×10^3 (F), DPPH and EPR assay parameters – see Fig. 1.

Induction and amplification of the signals of Fe-NO complexes with exogenous NO

To determine whether the lack of DNIC complexes in ascites of L5178Y lymphoma growing in DBA/2 hosts is due to low level of NO (generated as an effector substance in anti-tumor defence; Szczygiel *et al.*, 2004; Plonka, Plonka, Cieszka, Raczek & Lukiewicz, 1998; Bastian *et al.*, 1994), or due to the lack of non-heme iron, we treated white and red ascites from DBA/2 with exogenous NO. In red ascites the only effect of the addition of NO was a dose-dependent amplification of HbNO signals (Fig. 2 A, B), while DNIC signal could be artificially induced by a large excess of exogenous NO only in white ascites (Fig. 2C, D). The quantitative approximation of these phenomena showed that only in white ascites the amount of blood limits the appearance of HbNO signals. In fact, addition of 40 mM NO (the final concentration in the ascites) resulted in an increase of HbNO signal from 10 to over 10 000 a.u. in the red ascites, and from 0 to 68 a.u. in white ascites. 80 M (i.e. the 500-fold lower concentration) resulted in ca 24 a.u. for red and 10 a.u. for white ascites. While in the red ascites the increase of the HbNO signal intensity was really ca. 500-fold when the concentration of exogenous NO increased also 500-fold, in the white ascites the corresponding increase was as low as ca. 7-fold. Only under these circumstances the DNIC signal could be generated, clearly because of the low level of hemoglobin in the ascites. This is not surprising, as in many biological materials including smooth muscles (Geng, Petersson, Wennmalm & Hansson, 1994), and tumor cells (Bastian *et al.*, 1994), the heme targets become saturated with NO first, and only then the excess of NO is able to create EPR-detectable complexes with non-heme Fe. In the situation of high dominance of Hb over the non-heme Fe, the HbNO

signal being of 2-3 orders of magnitude higher than the DNIC signal makes the latter not detectable in red ascites. This is also the case of pure blood (Fig. 2E, F), in which it was never possible to induce DNIC signals despite the presence of spectacular HbNO signals, either by endotoxemia (Plonka, Chlopicki, Plonka, Jawien & Gryglewski, 1999b; Westenberger, Thanner, Ruf, Gersonde, Sutter & Trentz, 1990), or even by exogenous NO (our experiments -Fig. 2).

Concluding, although the exact content of non-heme iron, and its influence on the intensity of the observed signals has not been checked, it seems not to limit the appearance of DNIC NO complexes in ascites. The low level of NO is one, but not the only one reason for the lack of this type of the EPR signal in the ascites growing in DBA/2 mice. The second important obstacle to induce this signal is high amount of blood, as only in white ascites the DNIC signal could be artificially induced by the huge excess of NO.

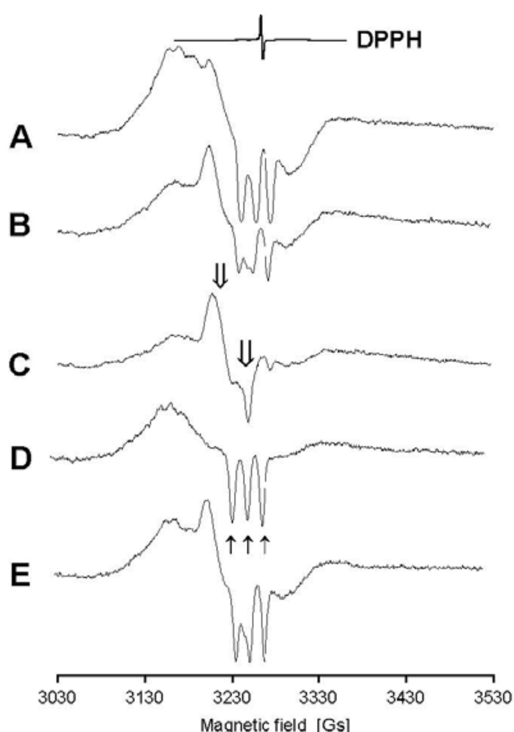

Fig. 3. EPR spectra of L5178Y lymphoma growing in the allogeneic outbred SWISS host. **A** and **B** – white ascites almost devoid of erythrocytes, **C** -similar ascites with a particularly pronounced DNIC signal (wide arrows), **D** – a pronounced HbNO signal (arrows) of red ascites highly supplemented with extravasate blood, **E** – a solid tumor. Instrumental gain: 2×10^{5} (A, B, C, D) and 1×10^{5} (E), DPPH and EPR assay parameters – see Fig. 1.

Appearance of Fe-NO complexes L5178Y lymphoma growing in Swiss hosts

In the allogeneic SWISS hosts some ascites revealed the DNIC signals beside HbNO, without addition of exogenous NO. It was the case for ca. 6% animals. The signals were detectable only in the ascites with low amounts of blood (ca. 50% of white ascites, cf. Fig. 3A-D; Tab. 1), and their amplitude was only ca. 1.5-fold bigger than the artificially induced DNIC signals in the ascites from DBA/2 hosts (84 a.u.). Both in tumors (Fig. 3 E), and in ascites (Fig. 3A-D) the presence of DNIC-type signals correlated with the intensity of Fe-NO complex signals, thus, indirectly -with the level of NO generation (Tab. 1) – the higher the average intensity of the signals, the bigger the percentage of tumors revealing the DNIC signals. This observation can be explained by the fact that Swiss mice are allogeneic hosts for L5178Y lymphoma, and reveal stronger antitumor response than DBA/2 hosts (Plonka *et al.*, 1999a, 1996b). The amount of NO generated in white ascites is enough to saturate their heme targets and to make complexes with the non-heme iron.

Interestingly, in Swiss hosts (Tab. 1) the intensity of HbNO signals in red ascites was lower than in white ascites ($0.05 > p \geq 0.01$). As in red ascites the level of endogenous NO was the main factor responsible for the intensity of the signals, we conclude that, indeed, in terminal stages of the disease, the increase in the level of blood in ascites must have been in a way correlated with the drop in NO generation.

Appearance of Fe-NO complexes in ascites and solid tumor of L5178 lymphoma

The distribution of erythrocytes in ascites is homogenous, so that practically every amount of NO, either generated physiologically or exogenous, can be trapped by "ascitic" hemoglobin revealing much higher affinity to NO than non-heme iron (Plonka *et al.,* 1999a; Plonka *et al.*, 1996a; Vanin, Serezhenkov, Mikoyan & Genkin, 1998; Bastian *et al.*, 1994; Geng *et al.*, 1994). Only in the situation of a very high level of NO generation, and a very low number of red blood cells, DNIC signals can be detected in ascites. This is the case of allogeneic Swiss hosts, where blood extravasation may take place at later stages of the disease (perhaps due to stronger anti-tumor defence than in DBA/2), and where the level of NO generation is much higher than in DBA/2 hosts, as judged by the intensities of the detected signals of Fe-NO complexes. This is also the reason for which the only way to observe DNIC signals in ascites of L5178Y lymphoma in DBA/2 hosts was the addition of big amounts of

exogenous NO to white ascites.

The presence of DNIC signals, sometimes strong ones, in the solid tumors (particularly in DBA/2 mice) indicates poor vascularization of some regions of the tumors – if the distribution of hemoglobin in tumor tissue were homogenous, the DNIC signals should not be detectable: the amount of erythrocytes in murine blood is ca. 6×10^9/ml (Brylinska, Kwiatkowska, Zaleska-Rutczynska, 1996), i.e. three times more than the amount detectable in the ascites with the highest amount of blood, where even the huge excess of exogenous NO (40 mM) could not induce DNIC signals. Such a high concentration of nitric oxide is never reached in physiological or even pathological conditions *in vivo* (Kosaka, Harada, Watanabe, Yoshihara, Katsuki & Shiga, 1992). These observations also indicate heterogeneity in the intensity of NO generation, as even in white ascites only the large excess of exogenous NO was able to generate these signals. There must be similar regions in tumors of poor blood supply and very high level of NO generation. On the other hand, judging by the percentage of tumors revealing DNIC signals, HbNO signals, or the lack of signals (Tab. 1), the appearance of such regions in DBA/2 hosts is lower than in allogeneic Swiss hosts, which remains in a logical agreement with the lower level of the antitumor response in DBA/2 than in Swiss mice.

CONCLUSIONS

Our experiments reveal that there are three main factors determining the appearance of Fe-NO complexes signals in L5178Y lymphoma:
(1) The level of NO generation, high in allogeneic, and low in syngeneic hosts. (2) Variable amount of blood in the ascites/tumor tissue. (3) The distribution of blood: homogenous in the ascites, and heterogenous in the solid tumor tissue.

In DBA/2 ascites DNIC signals are never present, because of the low level of NO, and relatively high level of blood, even in white ascites apparently devoid of blood. In Swiss mice only in a few percent of ascites the level of NO generation is high enough, and the amount of blood in ascites low enough to induce DNIC signals besides HbNO signals. In solid tumors of L5178Y-R lymphoma there are regions of a poor blood supply and strong NO production, where DNIC signals can be detected. In DBA/2 mice there are fewer such regions than in Swiss. The availability of non-heme iron seems not to play the important role in the appearance, but only in the intensity of the DNIC signals.

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