EXHALED NITRIC OXIDE AND NITRIC OXIDE SYNTHASE EXPRESSION IN HODGKIN'S DISEASE

G. GUIDA[1], B. CULLA[1], T. SCIRELLI[2], G. BELLONE[2], S. SCIASCIA[1,2], L. BRUSSINO[1], D. NOVERO[3], G. PALESTRO[1], E. HEFFLER[1], P. GAVAROTTI[3], G. ROLLA[1] and C. BUCCA[1]

[1]Department of Biomedical Sciences and Human Oncology, University of Torino; [2]Department of Clinical Physiopathology, University of Torino; [3]Azienda Ospedaliero Universitaria S. Giovanni Battista Hospital, Torino, Italy

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Hodgkin's disease (HD) is a malignant lymphoma with frequent mediastinal involvement, characterized by a significant inflammatory infiltration. Exhaled Nitric Oxide (F_ENO), is present in healthy humans, and has been proven to be increased in eosinophilic diseases such as allergic asthma. We investigated whether F_ENO is increased in mediastinal HD and whether NO is produced by lymphoma tissue. To this aim F_ENO was measured in 56 HD patients, 17 with and 39 without bulky mediastinal involvement, in the period from January 2007 to December 2008. Thirty-seven patients were reassessed after remission. Lymph node biopsies of 10 patients were evaluated for inducible (iNOS) and constitutive (eNOS) nitric oxide synthase expression by immunohistochemistry. F_ENO resulted significantly related to the mediastinal mass maximum diameter (p=0.009) and was significantly higher in patients with as compared to those without bulky mediastinal disease (38.7 ppb, $CI_{95\%}$ 19.3-58.0, versus 20.7 ppb, $CI_{95\%}$ 16.6-24.7; p=0.009). iNOS and eNOS immunoreactivity was observed in tumour and inflammatory cells (eosinophils and histiocytes). Only in patients with bulky mediastinal HD there was a significant decrease in F_ENO (from 50.4 ppb $CI_{95\%}$ 18.0-82.8 to 11.1 ppb $CI_{95\%}$ 4.4-17.8, p=0.011). In conclusion, high F_ENO and NOS expression in lymph-nodes indicate that NO is a component of the inflammatory network of HD. F_ENO may be proposed for the assessment and follow up of bulky mediastinal HD patients.

Nitric oxide (NO) is an intracellular and intercellular signalling molecule involved in the regulation of the immunological mechanisms, that, as free oxygen radical, takes part in inflammatory disorders (1). NO is synthesized from L-arginine by nitric oxide synthase (NOS) that exists in several isoforms: constitutive (eNOS) (endothelial NOS and neuronal NOS) and inducible (iNOS). Increased iNOS expression can be induced by immunostimulatory cytokines and has been found in many tumours, including B cell lymphoid neoplasms (2-3).

Hodgkin's disease (HD) is a malignant lymphoma originating from B cells, characterized by typical malignant cells, Hodgkin cells (HC) and Reed-Stenberg cells (HRS) (4). These cells represent less than 1% of the total tumour mass, and are surrounded by an extensive inflammatory infiltrate, characterized by lymphocytes, histiocytes, eosinophils, fibroblasts, neutrophils and plasma cells

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Mailing address: Prof. Caterina Bucca,
Dipartimento di Scienze Biomediche ed Oncologia Umana,
ASO S. Giovanni Battista – Molinette – Medicina Interna V,
Via Genova 3,
10126, Torino, Italy
Tel: ++39 0116336748 Fax: ++39 0116337238
e-mail: caterina.bucca@unito.it

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(5). A variety of cytokines and chemokines (i.e. IL5, IL-9, IL-10, IL-13, TNF-α RANTES, TARC) has been reported to be highly expressed by both HRS cells and Hodgkin's lymphoma cell lines (6) and by the surrounding inflammatory cells (7). Many cytokines promote iNOS and eNOS expression through activation of the transcriptional nuclear factor kB (NF-kB) in several immune, epithelial and tumour cells types (8). NF-kB has been reported to be constitutively activated in HRS cells and to promote the proliferation, survival and inhibition of apoptosis of HD cells (9-10). These observations suggest that NOS isoforms may be hyper-expressed in HD cells and in the surrounding inflammatory cells, and that, therefore, NO is largely produced in HD tissue.

NO is a diffusible gas mediator easily detectable in the exhaled air (F_ENO) (11) that is currently used as marker of airway inflammation in asthma (12). F_ENO has been reported to be increased in a patient with mediastinal bulky HD and to decrease after therapy (13).

The aim of this study is to assess whether F_ENO level may be used as a marker of mediastinal involvement in HD disease, that is whether F_ENO production is proportional to the extension of the mediastinal mass and decreases after disease remission. To ascertain NO synthesis by the tumour, iNOS and eNOS expression in lymph-nodes biopsies were assessed by immunohistochemistry.

MATERIALS AND METHODS

Subjects

The patients were selected from those who had received a new diagnosis of Hodgkin's Lymphoma with mediastinal localization (at least one pathological lymph node) at the haematology clinic in the period from January 2007 to December 2008. Exclusion criteria were history of asthma or allergic rhinitis, active smoking, current respiratory infections, or recent therapy with systemic corticosteroids in the previous two weeks.

Histological classification and disease staging were based on the World Health Organization Classification of Lymphoid neoplasms (14) and the Cotswolds meeting staging classification for Hodgkin's disease (15), respectively. Mediastinal bulky disease was defined when the mediastinal lymph node mass was larger than 9 cm in its greatest diameter or the ratio maximum mass transverse diameter/internal transverse thoracic diameter, measured on chest radiography (CXRs) at T5-T6 level, was 1/3 or greater. Blood samples were obtained for hemocromocytometric analysis with differential white blood cell count (WBC) and biochemical parameters including erythrocyte sedimentation rate (ERS). Hasenclever's score (International prognostic index – IPI) was calculated in advanced stage disease (16).

Treatment was established according to the European Society for Medical Oncology (ESMO) guidelines (17). The response assessment was defined according to International Working Group (IWG) guidelines (18-19).

Study design

Fifty-six consecutive patients were enrolled in the study. All underwent lung function tests and exhaled nitric oxide (F_ENO) at diagnosis. In a subgroup of 10 patients lymph node specimens were retrieved and processed for iNOS and eNOS expression, by immunohistochemistry. In 37 patients lung function tests and F_ENO were reassessed at least 6 months after remission.

All measurements carried out in the study, apart from F_ENO, are commonly performed in the assessment and follow-up of HD patients. The study was approved by the local ethics committee. All the patients gave their informed consent for the use of their data in the study.

Exhaled nitric oxide measurement

Fractional Exhaled Nitric Oxide (F_ENO) was measured according to ATS/ERS recommendations (20) with a NO chemiluminescent analyzer (Sievers NOA 280, Sievers, Boulder, CO, USA) using a sampling flow rate of 50 mL/sec. F_ENO values were calculated upon an end expiration plateau at least 3s long. The patient performed three acceptable trials and the mean value was registered. Sixty healthy subjects matched for age, sex and body mass index (BMI), served as controls for reference F_ENO values.

Lung function tests

Spirometry was performed using the computerized spirometer BAIRES (Biomedin, Padova, Italy). At least three maximal and repeatable flow-volume curves with vital capacity (VC) within 5% were recorded. From the curve with the greatest VC and maximal inspiratory and expiratory efforts, FEV_1, FEV_1/VC ratio, forced mid expiratory flow (FEF_{50}) and forced mid-inspiratory flow (FIF_{50}) and their ratio (FEF_{50}/ FIF_{50}) were calculated. VC, FEV_1 and FEF_{50} were expressed as percent of predicted (21). Gas transfer for carbon monoxide (DLCO) was measured using the single breath method.

Immunohistochemistry

Tissue specimens were obtained from stored

lymph nodes (6 supraclavear, 2 cervical, 1 axillary, 1 mediastinal) of ten HD patients. Formalin fixed paraffin-embedded tissue sections of 5 μm were deparaffinized in xylene baths and rehydrated in graded alcohol. For antigen retrieval the slides merged in 1X Target retrieval solution (Dako Corporation, Carpinteria, CA) were heated in a pressure cooker for 3 minutes. To block endogenous peroxydase activity, sections were incubated in 3% hydrogen peroxide buffer solution for 25 min. Non-specific antibody binding was blocked by pre-treatment with a solution of bovine serum albumin (0.1%) in fetal calf serum diluted 1:10 in PBS for 20 min. The sections were incubated overnight with the following primary antibodies: polyclonal rabbit anti-human iNOS diluted 1: 50 (Lab Vision Corporation, Fremont, CA) and mouse IgG1 monoclonal antibody eNOS/NOS type III clone 3 diluted 1:50 (BD Biosciences, San José, CA). Slides were then incubated with secondary anti-rabbit or anti-mouse IgG antibodies for 30 min followed by staining with peroxidase complex EnVision Plus System (Dako Corporation, Carpinteria, CA). The slides were finally counterstained with Mayer's hematoxylin for 5 sec, dehydrated, and mounted in Clarion (Biomeda, Foster City, CA). Negative controls were treated omitting the primary antibodies. Positive controls were lung and human endothelial for iNOS and eNOS respectively. Five non-neoplastic lymph nodes were used as further control on lymphoid tissue.

Evaluation of staining

Sections were blindly evaluated by two investigators. iNOS and eNOS stain was evaluated in tumour (HC and HRS) and reactive cells (eosinophils, histiocytes, lymphocytes) and localized within the cell cytoplasm and/ or nucleus. Stain images were captured using a LEICA DMLB light microscope. The degree of immunostaining was evaluated using the immunoreactive score (IRS) proposed by Remmele and Stegner (22) in which IRS corresponds to SI (staining intensity) x PP (average percentage of positive cells in the selected field). SI was scored as 0 = negative; 1 = weak; 2 = moderate; and 3 = strong. PP was scored as 0 = negative; 1 = 1-20% positive cells; 2 = 21-50% positive cells; and 3 = 51-100% positive cells. In each case 10 randomly selected visual fields were evaluated at a magnification x 630 from different areas of each specimen and the mean IRS was calculated for each cell type. A specimen was considered positive for a cell type if the mean IRS was ≥ 2.

Statistics

Data were analysed with the statistical SPSS software package (version 13.0 for Windows, Chicago, IL, USA). Normal distribution of variables were assessed according to Kolmogorov-Smirnov's test of normality. In case of non-normal distribution the variables were computed as logarithmic. Comparisons between continuous variables were estimated with unpaired and paired Student's t test or Mann-Whitney test, depending on the distribution of the variables. Categorical variables were compared with the Fisher's exact test. Correlations between parametric or non-parametric variables were obtained using Pearson's regression analysis, Spearman's rank correlation tests, and logistic regression analysis. A p value < 0.05 was considered to be significant.

RESULTS

Baseline Findings

Clinical characteristics

The 56 patients were 20 men and 36 women, mean age: 32 years (range 15-74). Disease staging showed: 10 patients in stage I (18%), 29 patients in stage II (52%), 9 patients in stage III (16%) and 8 patients in stage IV (14%). According to the histological classification, nodular sclerosis was found in 24 (43%) patients, mixed cellularity in 9 (16%), lymphocytic depletion in 14 (25%) and lymphocytic predominant in 9 (16%). Seventeen patients (30%) were classified as bulky Hodgkin's disease. Extra lymphoid disease was present in 5 patients: pleural or pericardial involvement in 3, lung or thorax in 2 patients.

Lung function tests and laboratory parameters

Lung function tests were normal in 45 patients (80%), showed restrictive defect in 8 patients all with bulky disease (14%), obstructive defect in 2 (4%) and combined restrictive and obstructive defect in 1 (2%).

Exhaled nitric oxide

At diagnosis, F_ENO was increased in most of the HD patients as compared to normal subjects (mean value 26.1 ppb, $CI_{95\%}$ 19.6-32.6 ppb *vs* 13.5 ppb; $CI_{95\%}$, 8.2-18.8 ppb; p=0.023). F_ENO was significantly directly correlated to the mediastinal mass maximum diameter (r = 0.345, p=0.009), and it was independent of gender, BMI, disease stage, presence of B symptoms, histological subtypes, ESR value, WBC and eosinophils in peripheral blood, IPI score, and lung function tests.

The characteristics of patients with and without

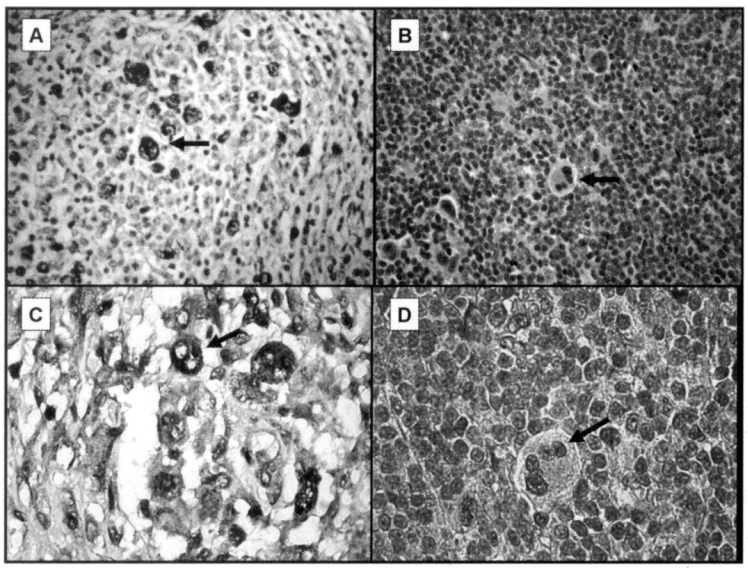

Fig. 1. *iNOS and eNOS expression in Hodgkin's disease. Panel **A**: iNOS is expressed by Reed Stenderg cells (black arrow) (Mayer's stain - original magnification ×250). Panel **C**: At high magnification (Mayer's stain - original magnification x630) iNOS is stained exclusively into the cytoplasm (black arrow). Panel **B**: eNOS is expressed by Reed Stenderg cells (black arrow) (Mayer's stain - original magnification ×250). Panel **D**: At high magnification Mayer's stain - (original magnification x630) eNOS is stained mainly into the nucleus (black arrow).*

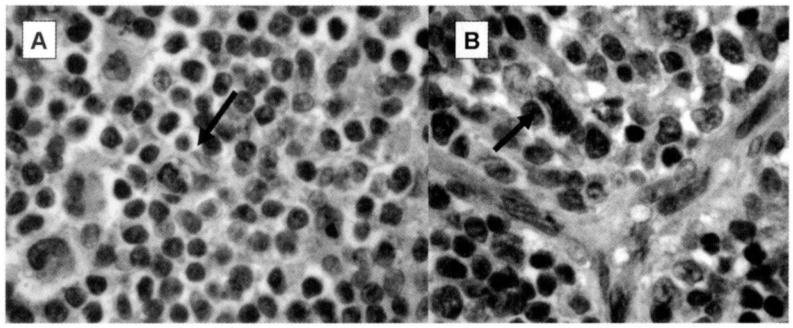

Fig. 2. *iNOS expression in eosinophils and in histiocytes in lymph nodes from Hodgkin's disease. Panel **A**: positive iNOS stain in one eosinophil (continuous arrow) within the neoplastic tissue (Mayer's stain - original magnification x1000). Panel **B**: positive iNOS stain in one histiocyte (dotted arrow) within the neoplastic tissue (Mayer's stain - original magnification x1000).*

mediastinal bulky disease are compared in Table I. Patients with bulky disease had significantly higher F_ENO value and lower VC and FEV1.

NOS immunostaining

The results of immunohistochemical analysis for iNOS and eNOS in lymph node biopsies from the 10 HD cases are reported in Table II. A positive immunoreactivity (IRS $\geq$ 2) was detected in all the specimens from the patients, but in none from the normal controls.

In all the cases, a positive immunoreactivity for iNOS was observed in cytoplasm of Hodgkin Reed-Stenberg cells and HC cells (Fig. 1, panel A, C). iNOS was also expressed in eosinophils and in histiocytes surrounding HD tumour cells (Fig. 2, panel A, B), but not in reactive lymphocytes and endothelial cells.

In all the samples, positive eNOS immunoreactivity, located mainly in the nucleus,

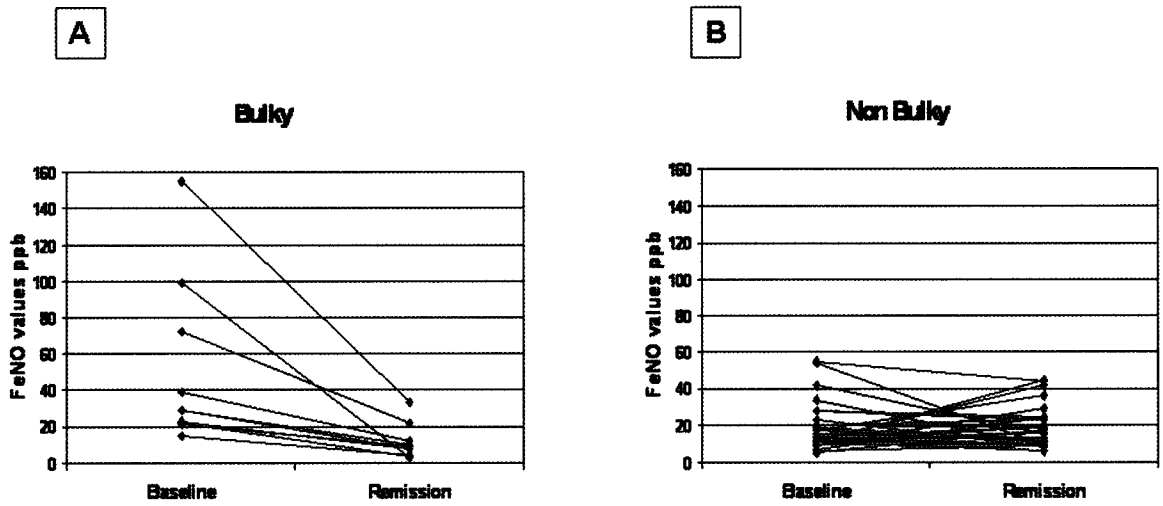

Fig. 3. *Individual F_ENO values before and after remission in patients according to bulky involvement. F_ENO values decrease to normal values from diagnosis to remission in most of HD patients with mediastinal bulky involvement (n=10, Panel A). No significant change is observed in patients without bulky involvement (n=27, Panel B)* .

was observed in HRS and HC cells (Fig. 1 panel B , D). eNOS was also expressed in histiocytes (10 patients), in endothelial cells (8 patients) and, to a lesser degree, in eosinophils (8 patients).

Effect of Treatment

Exhaled nitric oxide

Thirty-seven patients, 10 with and 27 without bulky disease, were reassessed after remission, obtained in 18 patients with chemotherapy alone (ABVD) and in 19 with combined adjuvant radiotherapy (RT).

In the majority of patients, a significant decrease in F_ENO (from 25.9 ppb $CI_{95\%}$ 19.3-32.5 ppb to 16.9 ppb $CI_{95\%}$13.1-20.7; p=0.035) was observed after remission due to the profound decrease in the patients with bulky mediastinal HD. F_ENO decreased significantly only in patients with bulky disease (from 50.4 ppb $CI_{95\%}$ 18.0-82.8 to 11.1 ppb $CI_{95\%}$ 4.4-17.8, p=0.011), while it remained nearly unchanged in patients without bulky disease (from 19.2 ppb $CI_{95\%}$ 13.9-24.5 to 19.0 ppb $CI_{95\%}$ 14.6-23.5, p=0.954). The individual F_ENO values measured before and after treatment in the two groups of patients are seen in Fig. 3.

DISCUSSION

The results of this study show that exhaled nitric oxide (F_ENO) is increased in patients with Hodgkin's disease and its production is proportional to the extension of the mediastinal mass. The finding that F_ENO was highest in patients with bulky disease and that it decreased significantly after disease remission only in patients with extensive mediastinal involvement, suggests that F_ENO originates in the tumour mass. This hypothesis is supported by the results of immunohistochemical analysis, which demonstrate that both the enzymes that synthesize NO, iNOS and eNOS are expressed in HD lymph node biopsies, either in tumour cells (Hodgkin Cells/Reed Stenberg cells) or in inflammatory cells (eosinophils and histiocytes).

To our knowledge, this is the first report on NOS expression in Hodgkin's Lymphoma. Exhaled NO may originate, by direct diffusion, from tumour and inflammatory cells. Alternatively, NO production by bronchial epithelial cells might be upregulated by the cytokine network deriving from the mediastinal lymphoma mass. We could not obtain bronchial or

Table I. *Comparison between HD patients with and without Bulky involvement.*

	Non-Bulky HD (N=39)	Bulky HD (N =17)	p
Sex (M/F)	14/25	6/11	0.996
Age, [range]	33 [15-74]	29 [17-50]	0.336
ERS*	52.1 (37-67)	50.6 (22-79.2)	0.379
WBC[â€] x 10^3/µL	7.440 (6.380-9.501)	9.927 (6.365-13.490)	0.059
Eosinophils x 10^3/µL	1.655 (1.185-2.125)	2.181 (0.345-4.018)	0.403
Hb[â€¡] g/dl	12.4 (11.7-13.1)	12.8 (11.7-14.0)	0.493
VC§	97 (91.7-103.1)	82.3 (74.8-89.9)	**0.003**
FEV_1‖	98.8 (93.7-104.0)	82.9 (73.7-92.1)	**0.002**
FEF_{50}¶	84.9 (76.1-93.6)	72.2 (58.9-85.4)	0.103
DLCO#	4.07 (3.53-4.60)	4.04 (3.62-4.46)	0.940
F_ENO (ppb)	20.7 (16.6-24.7)	38.7 (19.3-58.0)	**0.009**

Values are expressed as mean and $CI_{95\%}$. Spirometric values are expressed as percent of predicted values. Significant p value are reported in bold.
**ERS: erythrocyte sedimentation rate; [â€] WBC: white blood cell ; [â€¡] Hb: haemoglobin; §VC: vital capacity; ‖FEV_1: forced expiratory volume at 1st second.; ¶FEF_{50}: forced mid expiratory flow; #DLCO: gas transfer for carbon monoxide.*

Table II. *Expression of iNOS and eNOS in cells subtypes of 10 HD lymph node tissue biopsies.*

Cell type	iNOS		eNOS	
	Positive cases	**IRS**	**Positive cases**	**IRS**
Reed Stenberg / Hodgkin cells	10	4.3 ± 1.3	10	5.9 ± 1.7
eosinophils	10	4.7 ± 1.6	8	2.2 ± 1.6
histiocytes	10	5 0 ± 1.0	10	4.5 ± 1.6
lymphocytes	0	1.5 ± 0.4	1	0.3 ± 1.4
endothelium	0	0	8	4.8 ± 1.7

IRS = mean (± SD) immunoreactive score (see explanation in materials and methods section)

mediastinal biopsy from our patients in order to confirm this hypothesis.

The expression of NOS isoforms in HRS cells is consistent with the constitutive activation of NF-kB (8), which regulates the expression of many chemokines and cytokines. These molecules induce the reactive infiltrate of eosinophils, Th2 cells, and fibroblasts characteristic of HD, and may also mediate the growth and survival of HRS cell lines (5).

The role played by NO in lymphoma cells is still unclear (23). NO is considered an autocrine as well as a paracrine signal molecule, with positive effects on tumour progression (24). At relatively low concentration, NO is involved in tumour angiogenesis (25), proliferation of tumour lymphocytes (23), and protection of tumour cells from apoptosis (26). The constitutive expression of iNOS has been associated to tumour growth in T cell-leukemia (27), in B cell chronic lymphocytic leukaemia (28) and in B-cell non-Hodgkin's lymphoma (2). By contrast, at high concentration NO may induce apoptosis and slow down tumour growth (29). This effect has been mainly associated to NO production by iNOS in activated macrophages of neoplastic tissues (30). In our biopsy samples, high expression of iNOS was actually observed in histiocytes and eosinophils surrounding HRS cells.

In conclusion, the results of this study suggest that nitric oxide is produced by the cellular components of Hodgkin's disease. Measurement of NO in the exhaled air may be a useful and non-invasive method to evaluate airway inflammation in HD patients with bulky mediastinal disease. The lack of any correlation between FE_{NO} and disease staging, suggests that increased FE_{NO} reflects only the loco regional extension of the disease. Further studies are needed to confirm the role of F_ENO in the monitoring of HD and in predicting disease remission.

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