Jpn. J. Cancer Res. 85, 384–388, April 1994

Lethal Midline Granuloma in Okinawa with Special Emphasis on Polymorphic Reticulosis

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Lethal midline granuloma (LMG) is a clinical term used to describe a condition which may be manifested histologically as Wegener's granulomatosis (WG), polymorphic reticulosis (PR), and malignant lymphoma (ML). WG is an inflammatory disease, and PR and ML are considered to represent a neoplastic proliferation of lymphoreticular cells. In this report, twenty-two cases of LMG in Okinawa were examined. The frequency of LMG per 100,000 outpatients of the ear, nose and throat clinic in Okinawa was 67, and the higher frequency of PR (27) and ML (34) in Okinawa than in other districts of Japan was characteristic. Polymerase chain reaction, *in situ* hybridization, and immunohistochemical studies showed that the proliferating cells in PR were $CD43^+$ and simultaneously contained Epstein-Barr viral genome in their nuclei. The higher frequency of PR and ML in Okinawa is discussed in conjunction with a review of pertinent literature: multiple factors including genetic, viral environmental, and socioeconomic factors seem to affect the frequencies of these diseases.

Key words: Epstein-Barr virus — Wegener's granulomatosis — Polymorphic reticulosis — Malignant lymphoma — Epidemiology

Lethal midline granuloma (LMG) is a clinical term for the disease showing a progressive, necrotic change in the upper respiratory tract, especially the nasal cavity. Pathologically, LMG is composed of Wegener's granulomatosis (WG), polymorphic reticulosis (PR) or midline malignant reticulosis (MMR), and ordinary malignant lymphoma (ML). PR and ML are considered to represent a neoplastic proliferation of lymphoreticular cells. A previous study suggested that the frequency of each type of LMG is different between Eastern and Western countries: WG is the commonest disease in Western countries,[1] while PR is commonest in Eastern countries (the incidence in Asian countries is three to 15 times higher than that in England). The frequency of PR differs according to district even within a single country; it is much higher in Kyushu, the southwestern district of Japan, than in other districts of Japan.[1,2]

Okinawa prefecture, an area in which T-cell lymphoproliferative disease associated with human T-cell leukemia virus-I (HTLV-I) infection is endemic, is a group of islands in the southwestern region of Japan, between Kyushu district and Taiwan. Genetical differences between Okinawan people and other Japanese have been reported.[3] We have conducted pathological and epidemiological studies on LMG in Okinawa. Since a recent study suggested an etiological role of Epstein-Barr virus (EBV) in the development of PR,[4,5] we also examined the involvement of EBV by means of polymerase chain reaction (PCR) and *in situ* hybridization studies.

MATERIALS AND METHODS

Twenty-two patients with LMG treated at the Department of Otorhinolaryngology, University of the Ryukyus during the period of 1973 to 1991 were reviewed. All patients were natives of Okinawa. These patients presented with a nasal obstruction, nasal pain, or nasal bleeding frequently accompanied with fever. Physical examination revealed a necrotic destruction of nasal septum or turbinate which extended to the upper respiratory tract. Pathological specimens obtained from the patients were fixed in 10% formalin and embedded in paraffin. All histologic sections were reviewed by one of us (K.A.). The diagnosis of WG was based on the presence of multinucleated giant cells, which tended to be grouped near the blood vessels, and/or necrotizing vasculitis in the upper respiratory tract. The diagnosis of PR was indicated by the polymorphous character of cellular infiltrates consisting of large, atypical, mono- or multinucleated cells, together with normal-appearing inflammatory cells (Fig. 1). Cases showing monomorphous proliferation of lymphoreticular cells were diagnosed as ordinary lymphoma. Cases showing infiltration of small lymphoid cells, plasma cells, and macrophages without

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large atypical cells or findings suggestive of WG, were categorized as chronic inflammation. Twenty-two cases were registered as LMG. The total number of new patients visiting the ear, nose and throat (ENT) clinic during the same period was 32,874: this number was used to calculate the relative incidence of LMG.

Immunohistochemical study Tissue immunohistochemistry (avidin-biotin-complex method) was carried out in 19 cases, 9 with PR and 10 with ML. In brief, the sections were (1) incubated with normal horse serum for 30 min, and (2) incubated overnight at 4°C with mouse anti-human monoclonal antibodies Mx-Pan B (CD20) (Kyowa Medex, Tokyo), MB-1, MT-1 (CD43) (Bioscience, Emmenbruke, Switzerland), and UCHL-1 (CD45RO) (Dakopatts, Copenhagen), diluted at 1:100.

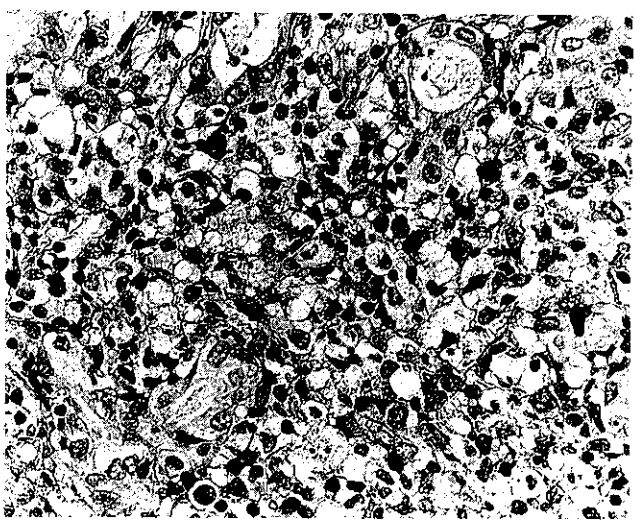

Fig. 1. Histology of polymorphic reticulosis showing polymorphic infiltrates. Large atypical cells intermingled with medium to small lymphoid cells and macrophages. Hematoxylin and eosin stain, ×300.

Subsequent reactions were carried out by using the Vectastain kit Lot No. PK-4001 (Vector, Burlingame, CA). The peroxidase reaction was completed in phosphate-buffered saline (pH 7.4) containing 0.005% H_2O_2 and 0.003% 3,3′-diaminobenzidine tetrachloride. Antibodies CD20 and MB-1 are directed to human B-lymphocytes and CD45RO and CD43 to T-lymphocytes. In six cases with PR positive for EBV genome by PCR, paraffin-embedded sections were stained with monoclonal antibody EBV CSI-4 (Dakopatts, diluted at 1:50), which recognizes the EBV-encoded latent menbrane protein (LMP)-1. Paraffin sections were treated with microwaves for five minutes in 0.01% citrate buffer before incubation with the antibody. Paraffin-embedded or acetone-fixed cytospin specimens of Raji cells and Ramos cells were used as positive and negative controls, respectively.

PCR The presence of EBV and HTLV-I sequences was examined by PCR in 9 cases with PR. DNA was extracted from formalin-fixed, paraffin-embedded tissues, and was used for the PCR, which was performed using heat-stable *Thermus thermophilus* DNA polymerase (Toyobo, Osaka). Primers and probes were synthesized by a DNA synthesizer (model 391, Applied Biosystems, Foster, CA) and purified in an Oligonucleotide Purification Cartridge (Applied Biosystems). The sequences of primers and probes are shown in Table I. Primers of EBV were located in the long internal repeat 1, according to Uhara *et al.*[6] Primers for HTLV-I were selected in the pX region. The PCR products were electrophoresed on a 2% agarose gel and visualized under ultraviolet light after staining with ethidium bromide. The products were transferred onto a nylon membrane and hybridized with the ^{32}P-end-labeled specific oligonucleotide probe. The positive control for detection of EBV genome by the PCR method was Raji cells, an EBV-positive Burkitt's lymphoma cell line. Ramos cells, derived from EBV-negative Burkitt's lymphoma cells, were used as the negative control for EBV. The Raji and Ramos cells were provided by the Japanese Cancer Research Resources Bank (Tokyo).

Table I. Sequences of Oligonucleotide Primers and Probes

		Sequence	Genomic location
EBV	Primer 1	5′-CCAGA CAGCA GCCAA TTGTC-3′	1087–1106
	Primer 2	5′-GGTAG AAGAC CCCCT CTTAC-3′	1196–1215
	Probe	5′-CCCTG GTATA AAGTG GTCCT-GCAGC TATTT CTGGT CGCAT C-3′	1131–1215
HTLV	Primer 1	5′-CAGGG TTTGG ACAGA GTCTT-3′	7311–7330
	Primer 2	5′-AACTG TAGAG CTGAG CCGAT-3′	7487–7506
	Probe	5′-ATCTC TGGGG GACTA TGTTC-GGCCC GCCTA-3′	7388–7417

Table II. Sequences of 12 Sets of Primers in *Bam*HI-W of EBV

	Sequence	5′----------3′				A:C:G:T
1	1–20	GATCC	CCCCA	CCGGC	CCTTC	2:12:3:3
	115–134	AAGGC	GACTC	GCCCG	GGCTG	3:7:8:2
2	139–158	CTCCC	CTGGC	CTCTC	CTTCC	0:12:2:6
	247–266	AGAGA	CTGGG	CGGCT	GCAGG	4:4:10:2
3	279–298	GGGCG	CCAGC	TTTTC	TCCCC	1:9:5:5
	384–403	TGTCC	ACCGT	GGGGA	GGGGT	2:4:10:4
4	423–442	CCATC	CCCGC	CCCCC	TGTGT	1:12:3:4
	544–563	GGGCC	GCCAG	GGGGG	CAAAA	5:5:10:0
5	572–591	CCCCG	GGCGC	CCCCA	AACTT	3:11:4:2
	696–715	GCCTG	GTGAC	AGGGC	GCGCA	3:6:9:2
6	883–902	CCACG	CGCGC	ATAAT	GGCGG	4:7:7:2
	984–1003	GCCCA	CTCCC	CTGTC	TGGGG	1:9:6:4
7	1330–1349	CCATC	CAAGC	CTAGG	GGAGA	6:6:6:2
	1428–1447	GGTGG	AGTGT	TGGGC	TTAGC	2:2:10:6
8	1451–1470	CCCAG	GCACA	CACTA	CACAC	7:10:2:1
	1559–1578	GACGA	GGACC	CTTCT	ACGGA	5:6:6:3
9	1585–1604	GAAGA	GGAGG	TGGTA	AGCGC	6:2:10:2
	1697–1715	TAATC	CCACC	CAGAC	TAGCC	6:9:2:3
10	1742–1761	GGCTT	CATGC	CCTCC	TCAGT	2:8:4:6
	1851–1870	CACAT	GTGTC	CAGGC	TGTGG	3:5:7:5
11	1881–1900	TGGCC	TCTAA	GGCCC	TCGGG	2:7:7:4
	1981–2000	GGACC	GGGGG	AGGAT	CAGGA	5:3:11:1
12	2633–2652	CTGGC	GCCTG	CTCGG	GGCCA	1:8:8:3
	2733–2752	CCAGG	AGGGC	GCCTG	GAGGC	3:6:10:1

EBV sequences were detected in the Raji cells but not in the Ramos cells. As positive controls for HTLV-I, two specimens of lymph node involved by adult T-cell leukemia (ATL) were examined. Serum titers of anti-HTLV-I antigen in these two patients were 2,500 and 40,000, respectively.

***In situ* hybridization** We have established a highly sensitive *in situ* hybridization procedure using digoxigenin-11-dUTP-labeled probes which were prepared by PCR.[7] With the use of twelve sets of primers, the *Bam*HI-W fragment of the EBV was amplified with labeled substrate in individual PCR. The sequences of the primers are listed in Table II. Then the 12 probes (Table III) with an average size of 120 base pairs, were mixed together and hybridized with the histologic sections. The specificity of the probes and the staining was evaluated by means of the following control studies; (1) the slides were incubated with the hybridization buffer containing unlabeled DNA, then incubated with anti-digoxigenin antibody-alkaline phosphatase conjugate and its substrate, (2) signals were observed using digoxigenized probe for JD repetitive sequence of mouse gene. This probe was prepared by random primed labeling as described above.

Double-labeling immunohistochemical and *in situ* hybridization studies performed on formalin-fixed paraffin-embedded sections. The immunohistochemical studies using alkaline phosphatase and mouse monoclonal anti-alkaline phosphatase were performed first with the monoclonal antibody CD43, followed by the *in situ* hybridization studies.

Table III. List of Probes Prepared by PCR

Probe	Position (bp)	Length	G/C (%)
1	1–134	134	71
2	139–266	128	76
3	279–403	125	69
4	423–563	141	68
5	572–715	144	72
6	883–1003	121	61
7	1330–1447	118	61
8	1451–1579	129	64
9	1585–1716	132	52
10	1742–1870	129	63
11	1881–2000	120	74
12	2633–2752	120	74

RESULTS

Frequency of LMG Frequency, age distribution, and sex ratio of LMG are summarized in Table IV. All but two cases were neoplasia of lymphoreticular cells, nine of polymorphic reticulosis and 11 of ordinary lymphoma. Patients with PR were younger than those with ML. A

Table IV. Age, Sex and Frequency of LMG Patients in Okinawa (1973–1991)

Disease	Number of patients (Frequency per 100,000 ENT patients)	Age		Sex (male/female)
		Range	Median	
WG	1 (3)	43		1/0
PR	9 (27.4)	34–73	33.7	6/3
ML	11 (33.5)	8–85	54.8	7/4
CI	1 (3)	26		1/0

WG: Wegener's granulomatosis, PR: polymorphic reticulosis,
ML: ordinary lymphoma, CI: chronic inflammation

Table V. Immunohistochemical Study of Lethal Midline Granuloma

Disease	Number of cases		
	$CD45RO^+/CD43^+$	$CD20^+/MB\text{-}1^+$	$CD45RO^-$, $CD43^-$, $CD20^-$, $MB\text{-}1^-$
PR	8	0	1
NHL	2	6	2

preponderance of male patients with PR or ML was seen. Frequencies of WG, PR, ML and chronic inflammation per 100,000 ENT patients were 3, 27.4, 33.5, and 3, respectively.

PCR The EBV genome was detected in 6 (67%) of nine cases with PR. HTLV-I genome could not be detected in any case.

Immunohistochemical study The results of immunohistochemical study are shown in Table V. Eight out of nine cases with PR were positive for CD43 and/or CD45RO and none showed a positive reaction for CD20 and MB-1. In cases with malignant lymphoma, six out of eleven were positive for CD20 and/or MB-1. Two cases were positive for CD43 and CD45RO but negative for CD20 and MB-1. Proliferating cells in all six cases with PR positive for EBV by PCR were positively stained by LMP-1.

***In situ* hybridization for EBV genome** *In situ* hybridization procedures for EBV genomes were carried out in six cases with PR positive by PCR. Positive signals were observed in the large atypical cells of all cases but one. Double-labeling immunohistochemical and *in situ* hybridization studies revealed the EBV DNA-positive cells to be positive for CD43 (Fig. 2).

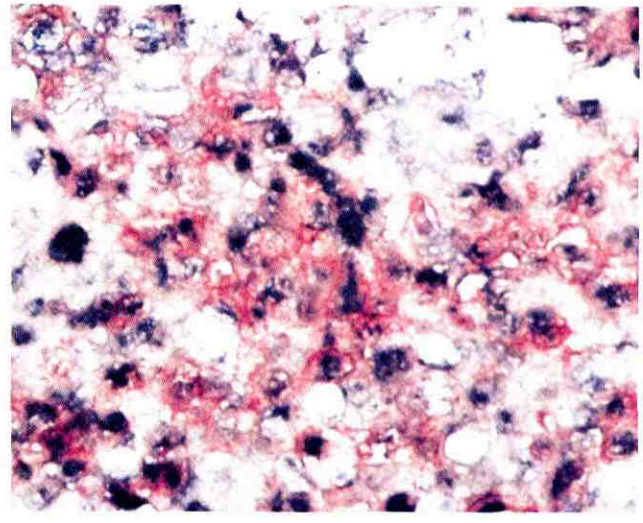

Fig. 2. Double-labeling immunohistochemical and *in situ* hybridization showing the expression of CD43 (red) in many EBV DNA-positive (blue-black) cells. ×900.

DISCUSSION

A nationwide study on LMG in Japan revealed clear differences in the frequency of each type between Eastern and Western countries.[1] When compared to Seoul, Shanghai, and Japan excluding Okinawa,[2] in which the frequencies of WG, PR and ML were 0, 40.8, and 10.9, 0, 9.8, and 7.2, and 4, 8, and 6, respectively, a relative low frequency of WG and a much higher frequency of ML were evident in Okinawa. The present study revealed a higher frequency of PR and ML in Okinawa than in Japan excluding Okinawa or in Shanghai, China. The southwest island district of Japan is an ATL endemic area.[8,9] The present immunohistochemical study revealed that tumor cells were stained with monoclonal anti T-cell antibodies, CD43 and/or CD45RO. This is in agreement with the previous immunohistochemical

study, in which the term "nasal T-cell lymphoma" was proposed for PR. This might suggest that the HTLV-I infection is a contributing factor to the high frequency of PR and ML in Okinawa. However, the PCR study did not show the HTLV-I genome in the PR lesion, ruling out a contribution of HTLV-I to the development of PR.

The present PR cases were identical with cases previously reported as nasal T-cell lymphoma presenting as LMG (NTL-LMG).[4,5] The majority of NTL-LMG cases, however, failed to demonstrate T-cell receptor gene rearrangements.[10] It is suggested that NTL-LMG is a neoplasia of natural killer cells.[11]

After infection of normal lymphoid cells with EBV, which is ubiquitous throughout the world, the EBV genome persists in the cells. An etiological role of EBV in the development of certain types of malignant lymphoma such as Burkitt's lymphoma and nasopharyngeal carcinoma has been postulated. Many Okinawans have latent EBV infection and there is a two-times-higher frequency of nasopharyngeal carcinoma in Okinawa compared with that of Japan excluding Okinawa (unpublished data). It seems very likely that EBV is a causative factor in the high frequency of PR and ML in Okinawa. Indeed, some authors have reported a close association between EBV and development of PR[12] or nasal T-cell lymphoma.[4,5] In the present study, the frequency of EBV genome in the PR lesions was 67% (6 of 9 patients) in Okinawa, which is higher than that in the Kinki district in Japan, 33%. These findings, together with the expression of LMP-1, suggest that the EBV infection could be a causative factor for PR, not merely coincidental.

The frequency of each type of LMG differs among Western and Eastern countries: Wegener's granulomatosis is more frequent in Western countries than in Eastern countries, whereas PR and ML are more frequent in Eastern countries than in Western countries.[1] One of the causes of this difference might be genetic.[1,2] Also, there is a possibility that socioeconomic factors affect the development of LMG.[1]

In conclusion, we consider that the higher frequency of PR and ML in Okinawa than in Japan excluding Okinawa can not be explained in terms of a single factor, but rather multiple factors including viral environmental, socioeconomic, and genetic factors may be involved.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for Cancer Research (4-5) from the Ministry of Health and Welfare and grants from the Ministry of Education, Science and Culture (04151019, 04770193, 04771418, 05152111), Japan.

(Received September 20, 1993/Accepted January 5, 1994)

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