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Epstein-Barr Virus Genome Carrying Lymphocyte Subpopulations of Human Palatine Tonsils[1] (39922)

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Introduction. The human palatine tonsil, a major lymphoid organ of the oropharynx, has received the focus of attention in relation to EBV localization in the head and neck. Serological studies of Tischendorf (1) first showed a relationship between EBV and heterophil-negative exudative tonsillitis. Later, our laboratory (2) supported and expanded these findings by reporting significant IgM-specific antibody titer changes for EBV occurring in patients with the same disease. Subsequently, Werner (3) experimentally inoculated gibbons intratonsillary with EBV and all animals contracted exudative tonsillitis and sero-converted following incubation. Evidence of the virus, Epstein-Barr nuclear antigen (EBNA), was then found by Veltri *et al.* (4) in the tonsil lymphocytes of patients with exudative tonsillitis, along with positive serology to EBV, early antigen (EA), and/or virus capsid antigen (VCA). The purpose of this investigation was to confirm the existence of a defined patient sample harboring latent EBV and to define specific subpopulations harboring EBV virus.

Materials and methods. Collection of tonsils. The right and left human palatine tonsils were obtained from 23 patients diagnosed as having recurrent exudative tonsillitis. The patients ranged in age from 4 to 29 years, with the mean age being 12 years. Following tonsillectomy, the tonsils were placed in Hanks' balanced salt solution (HBSS) containing 4 units/ml of heparin, 1000 units/ml of penicillin G, and 1000 μg/ml of streptomycin and then transported to the laboratory in an ice bath. A serum sample was also obtained at the time of tonsillectomy.

Preparation of tonsil lymphocytes (TL). The method used for the isolation and purification of TL is a procedure described by Sloyer *et al.* (5) and modified by Veltri *et al.* (4).

All 23 tonsils fractionated by this procedure yielded an average cell viability of 93%, as determined by trypan blue dye exclusion. By Wright's staining an average of 97% of the cells in the final TL populations were identified as lymphocytes.

Enumeration of the surface immunoglobulin (SIg)-bearing TL population. Tonsil lymphocytes bearing SIg (B lymphocytes) were detected by the method described by Jondal and Klein (6). Five-tenths of a milliliter of a 4×10^6-cells/ml suspension of TL were mixed with fluoroscein isothiocyanate-conjugated anti-human IgG, IgM, and IgA (Hyland Laboratories, Costa Mesa, Calif.). The cells were agitated thoroughly and then incubated at 37° for 30 min. A drop of this suspension was observed using a Leitz-UV-Ortholux microscope and those cells exhibiting membrane fluorescence were considered positive for the presence of SIg (4).

Preparation of erythrocyte antibody (EA) and erythrocyte antibody complement (EAC) complexes. Rabbit anti-SRBC serum (Hyland Laboratories, Costa Mesa, Calif.) was fractioned on a Sephadex G-200 column to obtain (IgG) and 19S (IgM) immunoglobulin fractions. Both fractions were concentrated and analyzed for purity by immunoelectophoresis.

The B-lymphocyte subpopulation of TL-bearing Fc receptors (erythrocyte antibody rosette formers) and C3 receptors (erythrocyte antibody complement rosette formers) were assayed by the method of Bianca *et al.* (7).

Enumeration of EA rosettes. A modification of the method of Siegel *et al.* was utilized (8). Equal volumes of the 0.5% EA suspension as prepared above and a 4

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$\times 10^6$ cells/ml suspension of TL were mixed. The cells were centrifuged at 100*g* for 5 min and incubated for 1 hr at 37°. Following incubation, the pellet was resuspended by agitation and a total of 200 lymphocytes was counted. Those TL binding four or more SRBC were considered positive for EA-rosette formation. Controls consisted of running the same procedure on the isolated B-lymphocyte subpopulation using unsensitized SRBC in the place of EA.

Enumeration of EAC rosettes. A modification of the procedure of Bianca *et al.* was used (7). Briefly, equal volumes of the 0.5% EAC suspension prepared as above and a 4×10^6 cells/ml suspension were mixed. Centrifugation, incubation, and rosetting were performed as in the above EA-rosetting procedure. The control utilized the isolated B-lymphocyte subpopulation and EAC prepared with heat-inactivated mouse serum.

Enumeration of E rosettes. The method utilized for the assay of T lymphocytes (E rosetting) in TL suspensions was a modification of the procedure described by Jondal *et al.* (6).

Isolation of tonsil lymphocyte subpopulations. The separation of lymphocytes based upon available receptors for either E–, EA–, or EAC–SRBC complexes from purified TL populations was achieved by first rosetting the subpopulations, then separating the more dense rosettes by isokinetic gradient centrifugation.

The E-rosette subpopulation was isolated from purified TL according to the method described above (6). The E rosettes were then layered onto precooled Ficoll–Paque gradients and centrifuged at 400*g* for 15 min at 4°. After centrifugation, these E rosettes (T lymphocytes) were collected from the pellet, the nonrosetted Tl were collected from the gradient interface, and these were mixed with EAC complexes. Such EAC rosettes were layered onto Ficoll–Paque gradients, and centrifuged at room temperature for 15 min at 400*g*. This B-lymphocyte subpopulation was then collected from the pellet, the nonrosetting TL were removed from the gradient interface, and the EA-rosette procedure was performed on the latter. After rosetting, the cells were placed on gradients as above and spun again at room temperature for 15 min at 400*g*. The EA-rosette, B-lymphocyte subpopulation was collected from the pellet and the nonrosetting TL, null lymphocytes, were collected from the gradient interface. The pelleted E rosettes were dissociated by incubation at 37° for 30 min and the EA and EAC were rosetted by vigorous agitation.

Slides were prepared from each of the four isolated lymphocyte subpopulations for EBV-coded antigen analysis (4).

Detection of EBV-coded antigen-producing lymphocytes. The detection of EBNA was accomplished by the anticomplement immunofluorescence test (ACIF) of Reedman and Klein (9). The EBV-positive P_3HR-1 cell line (10) was included as EBNA antigen-positive controls and CEM, a T-lymphocyte cell line, was used as an EBNA-negative control.

The method used for the detection of EA- and/or VCA-producing lymphocytes was the indirect fluorescent antibody test described by Veltri *et al.* (4). EA^+ VCA^+, EA^- VCA^+, and EA^- VCA^- $EBNA^-$ control sera kindly provided by Dr. Berge Hampar, Viral Carcinogenesis Branch, National Cancer Institute, Bethesda, Md., were used in this test.

Detection of serum antibodies to EBV antigens. The detection of serum antibodies to EA and VCA was accomplished by the indirect fluorescent antibody test described by Veltri *et al.* (2) and anti-EBNA antibodies were detected by the ACIF test of Reedman and Klein (9).

Results. Enumeration of TL subpopulations. Each of the 23 final TL populations was tested for the percentage of those lymphocytes possessing SIg or receptors for E, EA, or EAC complexes. Figure 1 illustrates the frequency and distribution of the various TL subpopulations. The T lymphocytes, all those cells forming E rosettes, comprised an average of $25.5 \pm 3.6\%$, with a range of 17.0–31.5%, of the total TL population. The overall B-lymphocyte population of the tonsil as identified by enumerating those cells possessing SIg was the largest lymphocyte population contained within the tonsil.

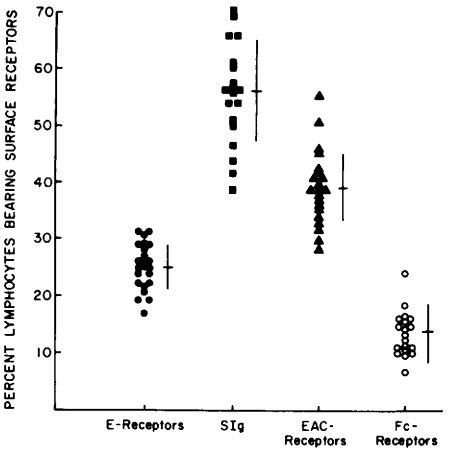

FIG. 1. Distribution of lymphocyte subpopulations of human palatine tonsils: (●) E rosettes (T lymphocytes); (■) surface bound immunoglobulin (SIg) receptors (B lymphocytes); (▲) EAC rosettes (B lymphocytes with C3 receptors); (○) EA rosettes (B lymphocytes with Fc receptors).

The mean was 56.8 ± 9.2%, with a range of 44.4–75.5%. These B lymphocytes were then further subdivided into two more B-lymphocyte subpopulations based on Fc and complement (C3) surface receptor markers. Of the 23 tonsils, 21 were tested for the presence of lymphocytes possessing the C3 receptor. An average of 49.5 ± 6.6%, with a range of 28.5–55.5%, of the TL from these 21 tonsils formed EAC rosettes. The smallest of the subpopulations was that composed of lymphocytes bearing the Fc receptor. Of the 23, 19 tonsils were tested for lymphocytes that had the capacity to form EA rosettes. An average of 13.6 ± 5.1%, with a range of 7.0–24.0%, of the TL possessed this particular receptor.

Detection of EBV antigen-producing lymphocytes. The total purified TL preparations of all 23 patients were screened for EBNA by the ACIF test. A positive test was indicated by finely granular fluorescence confined to the nucleus of the TL (4). Purified tonsil TL preparations of 17 of the 23 (73.9%) patients tested were positive for EBNA. Each TL preparation was also screened for the presence of EA and VCA. In each case all TL were found to be negative for these two EBV-coded antigens.

Each of the 17 EBNA-positive purified TL populations was then fractionated into the four subpopulations as mentioned previously, and each was retested for EBNA-positive lymphocytes. EBNA reactivity was localized almost exclusively in the C3- and Fc-bearing subpopulations. The TL bearing the C3 receptors showed the majority of the reactivity (Table I). All 17 EBNA-positive tonsils showed EBNA-bearing lymphocytes in the C3-receptor population. The percentage of EBNA-positive TL in this subpopulation ranged from 8 to 60% with a mean reactivity of 23.75 ± 14.86%. The B-lymphocyte subpopulation bearing the Fc receptor also contained EBNA-positive lymphocytes. Only 14 of the 17 positive tonsils were tested for EBNA reactivity in the Fc-receptor subpopulation and all were positive. The Fc-receptor-bearing subpopulation showed an average EBNA reactivity of 4.0% ± 2.08% with a range of 1–8%, much lower than that observed in the C3-receptor subpopulation. In all of the T- and null-lymphocyte populations studied, there were 0.4% or less EBNA-positive cells.

Detection of serum antibodies to EBV antigens. All 23 patients' sera were also screened for the presence of antibodies to EA, VCA, and EBNA. The results are shown in Table II. Of the 23 patients, 17

TABLE I. PERCENTAGE OF EBNA-CARRYING LYMPHOCYTES IN THE HUMAN PALATINE TL SUBPOPULATIONS.

Number of patients tested	Number EBNA positive	B lymphocytes[a] (%)	
		C3	Fc
21	17 (73.9%)	23.75 ± 14.86	4.0 ± 2.075

[a] Mean percentage of EBNA-positive cells in C3- and Fc-receptor subpopulations of B lymphocytes.

TABLE II. CORRELATION OF EBNA IN THE TONSIL LYMPHOCYTES TO EBV SEROLOGY.

Patients' TL-EBNA status	Serum antibodies to EBV antigens		
	EA (11)[a] (GMT)[b]	VCA (17) (GMT)	EBNA (17) (GMT)
17 positive	41.80	64.44	37.22
6 negative	<10.0	20.00	16.66

[a] Numbers in parentheses and numbers of sera positive at a 1:10 or greater dilution of the patients' sera.

[b] Geometric mean titers of all positive sera.

exhibited an anti-EBNA titer of 1:10 with a geometric mean titer (GMT) of 1:37. The same 17 patients showed positive anti-VCA reactivity with a GMT 1:64. Only 11 of the 23 patients exhibited serum antibodies to EA with a GMT of 1:42. All patients exhibiting EBNA-positive TL were also EBNA and VCA seropositive.

Discussion. Human TL isolated from 23 patients diagnosed as having recurrent exudative tonsillitis (RET) were subpopulated on the basis of three specific surface receptors, SRBC, Fc, and C3. We found a mean T-lymphocyte (E-rosette) population of 25.5 ± 3.6% in the 23 TL preparations. These values compare favorably to those we reported elsewhere (4) and to values reported by Dumont using electrophoretic mobility (11) and are similar to values reported for peripheral blood when the same techniques were employed (12–21). Such T lymphocytes appear to be immunologically reactive with respect to cell-mediated effector mechanisms (13, 14). The similarity of T-lymphocyte counts and effector functions both in the tonsils and in peripheral blood would suggest recirculation of these cells from peripheral blood and lymphatic circulation to the tonsils rather than local production.

The most significant difference between TL- and peripheral blood lymphocyte populations is an elevated B-lymphocyte count in TL (4, 8, 11, 13). Siegel demonstrated more B lymphocytes possessing C3 than Fc receptors in human palatine TL populations by a ratio of 3:1 (8). This is in contrast to the relatively equal concentrations of these two B-lymphocyte subpopulations found in the peripheral circulation (8, 15). These differences in total B-lymphocyte numbers as well as subpopulations in TL when compared to peripheral blood might suggest a local production mechanisms. Koburg, utilizing a tritiated thymidine label administered to rabbits via the internal carotid artery, provided experimental data to support local lymphopoiesis in palatine tonsils (16). These data and those of Lennert *et al.* (17, 28) using electron microscopy support the concept of the palatine tonsils as an active lymphopoietic organ. Hence, human palatine tonsils are an immunologically active lymphoid organ rich in B lymphocytes, possessing T lymphocytes, and capable of lymphopoiesis.

The observation of Jondal *et al.* (6) that EBV genome carrying lymphoblastoid cell lines are B lymphocytes was subsequently confirmed by Greaves *et al.* (18). Since the palatine tonsils are primarily B-lymphoid organs (4, 8) it is not inappropriate to suggest they might be the primary site for EBV infection and localization (4, 19). Our previous report indicated that 67% of purified TL preparations from patients with recurrent exudative tonsillitis possessed EBNA (4). The present study reports that 17 of 23 (73.9%) TL preparations carry EBNA. Furthermore, we found no evidence for synthesis of other EBV-coded antigens in any of the 23 patients' TL preparations. The latter observation would be quite consistent with the latent state for EBV which exists in the Raji lymphoblastoid cell line derived from a Burkitt's lymphoma patient (20), except that in human TL the numbers of EBNA-positive cells are much lower. A unique feature of our EBNA localization studies was the demonstration that the majority of EBNA reactivity is in the C3-receptor subpopulation. These data would tend to support Jondal and Klein's (21) recent observation of a close proximity between EBV receptors and C3d B-lymphocyte receptors. However, the low level of EBNA reactivity in the Fc B-lymphocyte subpopulation is left unexplained, but could be related to the dynamics of B-lymphocyte receptors on cells undergoing blastogenesis (15).

Another important facet of the finding of latent EBV in human tonsils relates to recent observations that TL preparations infected with EBV from the B95-8 and QIMR-NIL cell lines can transform such cells (23). However, in light of the data presented here, one might speculate that the transformation observed by these authors was in fact that result of activation of latent EBV by superinfection (24). Also their and our (unpublished data) inability to obtain spontaneous activation of TL preparations may be related not to small numbers of EBNA-positive cells as they suggest but, rather, to the presence of sen-

sitized killer T lymphocytes (14) in the tonsils which immediately destroy any spontaneously activated EBV-infected TL. We hope to eventually answer this question by employing the isolated subpopulations of TL.

The serological studies of EBV-coded antigens support the correlation between EBNA carrying TL and the history of exposure to EBV. The role of this latent state in the pathogenesis of RET and possibly other EBV-related diseases may be closer at hand with a well-defined patient sample.

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