Relationship between antibody productivity by activated human lymph node lymphocytes from lung cancer patients and lymphocyte subsets

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Abstract

Regional lymph node lymphocytes from five patients with primary lung cancer were analyzed for subset composition, and exposed *in vitro* to the polyclonal human B cell mitogen *Staphylococcus aureus* Cowan I (SACI) or the murine B cell mitogen lipopolysaccharide (LPS) and then fused with mouse myeloma cells for investigation at the clonal level of their antibody (Ab) production and its statistical relation to the original subset composition. No correlation was found between the proportion of CD19⁺, CD23⁺, or CD3⁺ cells in the lymphocyte sample prior to its exposure to either SACI or LPS, and the Ab production efficiency, defined as the ratio of the number of Ab producing wells to the total number of proliferating wells. For lymphocytes exposed to LPS, however, a strong correlation ($r = 0.931$, $p = 0.02$) was observed between the Ab production efficiency and the ratio of CD8⁺ to CD3⁺ cells (CD8/CD3) in the original sample at least within the ranges studied (CD8/CD3 = 0.216–0.288). For those exposed to SACI, no correlation was found between the Ab production efficiency and the CD8/CD3 ratio ($r = 0.881$, $p = 0.12$) or the proportion of CD8⁺ cells ($r = 0.808$, $p = 0.19$) in the original sample. These results suggest that the repertoire of B cells responsive to LPS is different at least in part from the repertoire responsive to SACI and that the ratio CD8/CD3 could serve as a practical predictor for Ab production by human lymphocytes stimulated with LPS.

Introduction

Peripheral blood lymphocytes (Croce et al., 1980; Imam et al., 1985), lymph node lymphocytes (Haspel et al., 1985; Kan-Mitchell et al., 1986; Kjeldsen et al., 1988), and spleen lymphocytes (Jahn et al., 1988) are commonly used as sources of B cells for the preparation of human monoclonal antibodies (mAbs). Regional lymph node lymphocytes from patients with cancer have been found particularly effective as B cell sources for the preparation of human mAbs capable of recognizing tumor-associated antigens (Siroka, 1987). Therefore, we employed such lymphocytes in the present analysis of lymphocyte subsets and antibody

(Ab) production levels, as one means of elucidating the relationship between Ab production efficiency and lymphocyte subsets. Ab production was measured at the clonal level, with heterohybridoma formed by the electrofusion of mouse myeloma cells and lymphocytes activated by *Staphylococcus aureus* Cowan I (SACI) (Forsgren et al., 1976; Ringdén and Rynnel-Dagöö, 1978; Ruuskanen et al., 1980; Schuurman et al., 1980) and by lipopolysaccharide (LPS) (Ho et al., 1985; Nozawa et al., 1989), both of which are known to stimulate Ab production by regional lymph node lymphocytes.

The ratio of Ab-producing hybridomas to proliferating hybridomas was investigated for correlation to lymphocyte subsets. The ratio was found to correlate with the ratio of CD8⁺ cells to CD3⁺ cells in

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the lymphocytes exposed to LPS, but not with the CD19⁺ or CD23⁺ cell content. The CD8/CD3 ratio may therefore be useful as a predictive parameter for Ab production efficiency of lymphocytes following their stimulation by LPS. In the case of lymphocyte stimulation by SACI, however, neither the CD8/CD3 ratio nor any subset content of the lymphocytes correlated with the Ab production by their hybridomas, and thus no apparent predictive parameter was found among the CD markers investigated.

Materials and methods

Agents

LPS (*Escherichia coli* 0127:B8) and hypoxanthine-aminopterin-thymidine (HAT) were purchased from Sigma Chemical Co. (St. Louis, MO). *Staphylococcus aureus* Cowan I (SACI) (Pansorbin cells 507861) was obtained from Calbiochem (San Diego, CA). Recombinant interleukin (IL)-6 was purchased from Genzyme Corp. (Cambridge, MA). Fetal calf serum (FCS) was obtained from Cytosystems (Catalog No. 15-010-0500V, Castle Hills, Australia). Bovine insulin, human transferrin, and sodium selenite were obtained from Cosmo-Bio Co. (Tokyo, Japan).

Lymphocyte preparation

Regional lymph nodes from patients with primary lung cancer were obtained aseptically during surgery as described previously (Yoshinari et al., 1995; Yoshinari et al., 1996). A single-cell suspension (lymphocytes) in 10% FCS-supplemented RPMI 1640 (Flow Laboratories) medium was prepared by passing the lymph nodes through sterile steel mesh (#200) after teasing with scissors.

Subset analysis

Subset analyses were done by FACS[®] at the Health Science Laboratories (Yokohama, Japan). The monoclonal antibodies (mAbs) used were: Leu-4 (CD3); Leu-3a (CD4); Leu-2a (CD8); Leu-1 (CD5); Leu-12 (CD19); Leu-20 (CD23); Leu-17 (CD38); Leu-19 (CD56); Leu-15 (CD11b); Leu-M3 (CD14); HLA-DR mAb.

In vitro stimulation of lymphocytes

Lymph node lymphocytes (2.5×10^6 cells per ml) pre-washed with phosphate-buffered saline (PBS) were resuspended in RDF medium (a 2:1:1 mixture of RPMI 1640, Dulbecco's modified Eagle medium (DMEM) and Ham's F12 media) with 10% FCS and 50 μ M 2-mercaptoethanol (2-ME). Each 2-ml portion of the cell suspension was pipetted into 6-well tissue culture plates, and mitogen (LPS, 20 μ g ml⁻¹ or SACI, 10 000⁻¹ (volume per volume)) was added to each well, followed by incubation at 37 °C for 5 d in humidified 5% CO₂ air.

Cell fusion and heterohybridoma incubation

Electrofusion of lymphocytes and mouse myeloma cells (P3X63Ag8.653; ATCC CRL 1580) was performed by the method of Fong et al. (1990) and Zimmermann et al. (1990) with some modifications. Briefly, mixed cells (lymphocytes:myeloma cells = $5 \times 10^6:5 \times 10^6$) were washed once with iso-osmolar fusion medium (300L3; 280 mM sorbitol, 0.1 mM Ca²⁺ acetate, 0.5 mM Mg²⁺ acetate, and 1 mg ml⁻¹ bovine serum albumin (BSA)). After collection by low-speed (1200 rpm $\times$ 10 min) centrifugation, the cell pellets were resuspended in 2.5 ml of hypo-osmolar fusion medium (75L3; 70 mM sorbitol, 0.1 mM Ca²⁺ acetate, 0.5 mM Mg²⁺ acetate, and 1 mg ml⁻¹ BSA). Five minutes later, 0.83-ml aliquots of the cell suspension were placed in fusion chambers and exposed to one rectangular electrical pulse (1 MHz, 1.00 kV per cm, 15 μ s) in a model SSH-1 cell fusion apparatus (Shimadzu, Kyoto, Japan). After standing for 5 min, each 0.83-ml aliquot (total volume; 2.5 ml) was pipetted into 30 ml of HAT medium (RDF medium supplemented with 100 μ M hypoxanthine, 16 μ M thymidine, 0.2 μ M aminopterin, 5 μ g ml⁻¹ bovine insulin, 5 μ g ml⁻¹ human transferrin, 5 ng ml⁻¹ sodium selenite, 50 μ M 2-mercaptoethanol, 20 U ml⁻¹ IL-6, 40% Balb/c mouse splenocyte culture supernatant, and 10% FCS) and then distributed in 0.1-ml portions into 96-well culture microplates. The plates were incubated for 2–4 weeks in humidified 5% CO₂ air at 37 °C. Wells with growing colonies were selected microscopically, production of secreted Ig was measured by sandwich enzyme-linked immunosorbent assay (ELISA), and Ig-secreting wells were maintained in hypoxanthine-thymidine (HT) medium (RDF medium supplemented with 100 μ M hypoxanthine, 16 μ M thymidine, 5 μ g ml⁻¹ bovine insulin, 5 μ g ml⁻¹ human transferrin,

5 ng ml⁻¹ sodium selenite, 50 μ M 2-mercaptoethanol, 20 U ml⁻¹ IL-6, and 10% FCS).

Measurement of Ig production

Human mAb was measured by sandwich ELISA, in which 96-well ELISA microplates (Sumitomo Bakelite, Tokyo) precoated overnight at 4 °C with 2000⁻¹ goat antibodies to human immunoglobulins (No. 4103, Tago, Burlingame, CA) were blocked for 1 h at room temperature with 2% BSA in PBS and washed twice with PBS. Subsequently the plates were incubated for 1 h at 37 °C in serial dilutions of 0.2% BSA-PBS of cell culture supernatants containing secreted mAb and were washed three times. Bound mAb was detected by incubation for 30 min at 37 °C with 2000⁻¹ peroxidase-conjugated goat antibody F(ab')₂ anti-human Ig (No. 3301-0231, Cappel, West Chester, PA) in 0.2% BSA-PBS. Plates were washed three times, followed by coloration with substrate (0.5 mg ml⁻¹ o-phenylenediamine, 0.015% H₂O₂ in 25 mM citrate-50 mM disodium phosphate buffer (pH 5.0)), and human mAb was quantitated using human IgM (No. 6001-1590, Cappel), IgG (No. 6001-0080, Cappel) and IgA (No. 6001-0020, Cappel) as the standards. Wells with a concentration of more than 20 ng ml⁻¹ of secreted Ig during at least three weeks were designated Ig-positive, and were counted.

Statistical analysis

Relative coefficients and significant *p* values were enumerated using StatFlex (ViewFlex Co.) statistical software.

Results

Lymphocyte subsets

The subset compositions of the lymphocytes from the five lung cancer patients, as analyzed by FACScan[®], are shown in Table 1 together with the mean and standard deviation for each subtype. The cancers were histologically classified adenocarcinoma in two patients and squamous cell carcinoma in the other three.

As shown, the mean value was 44.2±10.4% for cells showing a positive response for the B cell marker CD19, 14.6±3.3% for cells positive for the T cell marker CD8, a 0.6±0.2% for cells positive for CD14, which is reportedly LPS receptor (Wright et al., 1990; Ziegler-Heitbrock and Ulevitch, 1993).

Fused cell proliferation and Ab production

Table 2 shows the results on the cell proliferation and Ab (Ig) production, after 2–3 weeks of incubation of the hybridomas, which had been obtained by electrofusion of mouse myeloma cells P3X63Ag8.653 and the lymphocytes previously incubated for 5 d with either LPS or SACI. In the case of no addition of mitogens, hybridomas were hardly generated as reported previously (Yoshinari et al., 1996).

As indicated, the number of fused cells plated in each well was kept small (3 to 4 × 10⁴ lymphocytes per well) to minimize the occurrence of two or more colonies per well, and for this reason the percentage of wells with growth was at most 16.2% (No. 2, LPS), and the percentage of plated wells that showed Ab production (20 ng ml⁻¹ or more of secreted Ig after three weeks) was at most 4.2% (No. 2, SACI).

Microscopic observation showed that more than 98% of the wells with growth contained only one colony throughout the course of incubation. The number of wells with growth shown in Table 2 is the number containing single colonies, after the transfer of colonies from wells with two or more colonies as far as possible.

With lymphocytes that had been exposed to LPS, the percentage of wells with growth that exhibited Ab production was between 24 and 42%. With those exposed to SACI, the percentage was 33 and 72%. Ig incidence, defined as the ratio of the number of Ig producing wells to the number of lymphocytes plated, ranged from 0.85 × 10⁻⁶ (No. 4) to 1.9 × 10⁻⁶ (No. 3) with stimulation by LPS, and from 0.95 × 10⁻⁶ (No. 5) to 2.1 × 10⁻⁶ (No. 4) with stimulation by SACI.

Ab production efficiency and lymphocyte subsets

The coefficients of correlation between the Ab production efficiency and the lymphocyte subset content, as calculated for subsets of particular interest from the results shown in Tables 1 and 2, are shown in Table 3 and Figure 1. With stimulation by LPS, the Ab producing efficiency showed no correlation with the proportion of CD3⁺, CD8⁺, CD19⁺, or CD23⁺ cells, and a strong correlation (*r* = 0.931, *p* = 0.02) with the ratio of CD8⁺ to CD3⁺ cells in the lymphocytes (CD8/CD3).

It was not possible to calculate the coefficient of correlation for CD14⁺ cells, although they are of particular interest since they are reportedly an LPS receptor (Wright et al., 1990; Ziegler-Heitbrock and Ulevitch, 1993), because of their low incidence (0.2

Table 1. Subsets of regional lymph node lymphocytes from five lung cancer patients

No. Histology	1 (20MT) ^a Ad ^b	2 (26GO) Sq ^c	3 (27FO) Sq	4 (28SN) Ad	5 (29HY) Sq	mean	S.D.
CD3	60.2	53.7	56.9	73.2	39.3	56.7	10.9
CD4	41.9	40.9	39.1	58.1	30.3	42.1	9.0
CD8	17.3	11.6	16.4	18.1	9.8	14.6	3.3
CD5	76.3	74.4	57.0	81.8	62.4	70.4	9.2
CD19	40.6	48.8	42.0	29.0	60.8	44.2	10.4
CD23	34.0	38.0	16.3	24.1	53.2	33.1	12.6
CD38	33.9	32.4	N.T. ^d	N.T.	17.4	16.7	14.8
CD56	5.9	4.9	3.8	4.9	3.9	4.7	0.8
CD11b	28.0	27.8	28.5	18.9	38.3	28.3	6.1
CD14	0.7	0.7	0.2	0.9	0.6	0.6	0.2
HLA-DR	66.8	69.2	64.7	90.1	76.4	73.4	9.2

The subset composition of the lymphocytes from each patient is shown as the percentage of the lymphocytes showing a positive response for each marker by FACSscan[®] analysis, together with the mean and standard deviation for all five patients.

^a Patient identification number shown in parenthesis.

^b Ad = adenocarcinoma.

^c Sq = squamous cell carcinoma ^d N.T. = not tested.

to 0.9%). With SACI stimulation, the Ab production efficiency showed no significant correlation with CD8⁺ cells ($r=0.881$, $p=0.12$) or the ratio CD8/CD3 ($r=0.808$, $p=0.19$).

Discussion

The agents most commonly employed for *in vitro* activation of human lymphocytes are SACI (Forsgren et al., 1976; Ringdén and Rynnel-Dagöö, 1978; Ruuskanen et al., 1980; Schuurman et al., 1980), which is known as a polyclonal B cell activator, and PWM lectin (Jahn et al., 1988; Cairns et al., 1985; Umetsu and Geha, 1987), known as a T cell-dependent B cell activator. LPS, on the other hand, has been reported (Umetsu and Geha, 1987) to show little or no activating effect on human B cells, and particularly on purified B cells. These observations suggest that the effects of SACI and LPS on human lymphocytes may involve different pathways. Lymph node lymphocytes are known to consist of heterogeneous immune cells such as T cells, B cells, and monocytes. Therefore, we employed these two mitogens to test this hypothesis by investigating the relationship between lymphocyte subsets and the production of Abs by B cells following exposure of the lymphocytes to SACI and LPS, as exhibited at the clonal level by the heterohybridoma obtained by electrofusion of mouse myeloma cells

and the regional lymph node lymphocytes from lung cancer patients.

The subset analyses showed the lymphocytes to consist of 29.0 to 60.8% CD19⁺ cells (mean, 44.2%), 16.3 to 53.2% CD23⁺ cells (mean, 33.1%), and 9.8 to 18.1% CD8⁺ cells (mean, 14.6%). The values for CD8⁺ cells are similar to those found by Farzad et al. (1990) for CD8⁺ cells in lymph node lymphocytes from melanoma patients at stage I (mean 9.8%) and stage II (16.7%). The mean values for CD19⁺ cells found in our study are midway between those found in the study by Farzad et al. (stage I mean, 10.6%; stage II mean, 17.6%) and those found by Kipps and Duffy (1991) for tonsillar lymphocytes from tonsillitis patients (55 to 97%, mean 73%).

Since B cells and activated B cells are considered to consist almost entirely of CD19⁺ and CD23⁺ cells, respectively, it might be expected that the efficiency of Ab production (ratio of Ab producing wells to total proliferating wells) would be proportional to the proportion of CD19⁺ or CD23⁺ cells in the lymphocytes. In actuality, no such relationship was observed for either stimulating agent. In the case of LPS stimulation, a clear positive correlation with the Ab production efficiency was found only for the CD8/CD3 ($r=0.931$, $p=0.02$) ratio, suggesting that a portion of the B cells affected by CD8⁺ cells, presumably, particularly by CTLs, might show an activation response (whether direct or indirect) to LPS, with mediation by the cells

Table 2. Proliferation and Ab production of lymphocytes fused with mouse myeloma cells

No.	Histology	Mitogen	No. of cells ^a	No. of plates ^b	Wells with growth ^c	Wells with Ig ^d	Ig (%) ^e	Ig incidence ^f
1 (20MT) ^g	Ad	LPS	4×10^7	10	106	45	42	1.10×10^{-6}
2 (26GO)	Sq	LPS	13×10^7	50	778	189	24	1.40×10^{-6}
		SACI	13×10^7	50	616	201	33	1.50×10^{-6}
3 (27FO)	Sq	LPS	6×10^7	20	269	112	42	1.90×10^{-6}
		SACI	6×10^7	20	192	138	72	2.30×10^{-6}
4 (28SN)	Ad	LPS	6×10^7	20	217	51	24	0.85×10^{-6}
		SACI	6×10^7	20	186	129	69	2.10×10^{-6}
5 (29HY)	Sq	LPS	8×10^7	20	282	88	31	1.10×10^{-6}
		SACI	8×10^7	20	213	36	36	0.95×10^{-6}

^a Total number of lymphocytes plated, following 5 d exposure to mitogen and electrofusion with mouse myeloma cells.

^b 96-well plates, each well seeded with $3-4 \times 10^4$ lymphocytes.

^c Number of wells containing single colony after the division of a small number of wells showing two or more colonies.

^d Number of wells containing 20 ng ml^{-1} or more Ig.

^e Production efficiency: percentage of wells with growth that contained 20 ng ml^{-1} or more Ig.

^f Ratio of number of wells containing 20 ng ml^{-1} or more Ig to total number of lymphocytes plated.

^g Patient identification number shown in parenthesis.

Table 3. Correlations between Ig production efficiency^a and lymphocyte subsets^b

	Ig(%)–LPS	Ig(%)–SACI	CD8/CD3	CD3	CD4	CD8	CD19	CD23	CD56	CD14
Ig(%)–LPS	1.000	0.497 (0.51) ^c	0.931 (0.02)	–0.121	–0.380	0.341 (0.57)	0.024	–0.287	–0.023	–0.668
Ig(%)–SACI		1.000	0.808 (0.19)	0.616 (0.39)	0.495 (0.51)	0.881 (0.12)	–0.701 (0.30)	–0.798 (0.20)	–0.571	–0.285
CD8/CD3			1.000	0.140	–0.094	0.584 (0.30)	–0.243	–0.447	–0.026	–0.525
CD3				1.000	0.959 (0.01)	0.886 (0.05)	–0.993 (0.0005)	–0.747 (0.15)	0.185	0.390
CD4					1.000	0.740 (0.15)	–0.929 (0.02)	–0.594	0.181	0.562
CD8						1.000	–0.927 (0.02)	–0.818 (0.09)	0.153	0.077
CD19							1.000	0.801 (0.10)	–0.129	–0.296
CD23								1.000	0.365	0.316
CD56									1.000	0.706
CD14										1.000

^a Ig as shown in Table 2.

^b Proportion of lymphocytes showing positive response for given marker prior to exposure to mitogen, as shown in Table 1.

^c *p* Values are shown in parenthesis.

Figure 1. Plots of Ig production efficiency (Ig(%)) and marker-positive lymphocyte content in lymphocyte samples prior to exposure to SACI (closed circle) or LPS (open square), for the CD8/CD3 ratio and CD3⁺, CD8⁺, CD19⁺, and CD23⁺ cell contents. With stimulation by LPS, the Ab producing efficiency showed a strong correlation ($r=0.931$, $p=0.02$) with the ratio of CD8⁺ to CD3⁺ cells in the lymphocytes (CD8/CD3) while with SACI stimulation, the Ab production efficiency showed no significant correlation to the CD8/CD3 ratio ($r=0.808$, $p=0.19$).

(largely monocytes) that respond positively to CD14, which is reported to be an LPS receptor (Wright et al., 1990; Ziegler-Heitbrock and Ulevitch, 1993).

It should be also noted that in this case the proportion of B cells activated by LPS should be affected by the number of CD4⁺ cells present, and that the results of the present study did show a negative correlation with the ratio CD4/CD3.

In the case of SACI stimulation, however, the Ab production efficiency did not correlate significantly with the proportion of CD8⁺ cells in the lymphocytes or with the CD8/CD3 ratio, suggesting that direct re-activation by SACI of B cells affected by CD8⁺ cells (presumably, predominantly CTLs) leads to a repertoire of Ab-producing B cells that differs at least in part from the Ab-producing repertoire under LPS stimulation.

It may also be noted that the number of wells with growth was approximately 1.2 to 1.4 times as large

with exposure to LPS as that with exposure to SACI, possibly because a larger population of weakly activated B cells are produced with LPS than with SACI due to the indirect activation of B cells by LPS, considering from the fact that the amount of Ab produced by LPS stimulation was lower than that by SACI stimulation (data not shown). The mechanism of activation of lymph node lymphocytes by LPS, in any case, remains to be determined.

The results of the present study may have important practical implications. They indicate that, in practice, in the case of LPS stimulation it would be highly advantageous to use lymph node lymphocytes with a high population of CD8⁺ cells and a low population of CD4⁺ cells in order to obtain antibody-producing hybridomas. Furthermore, Steplewski and his colleagues (Tsujitani et al., 1995; Nakashima et al., 1996) have shown that *in vitro* stimulation of lymphocytes with IL-1, IL-2, IL-4 and IL-6 induces generation of tumor-

specific CTLs. It may be possible to combine their findings with our own, to obtain a system in which lymph node lymphocytes could be stimulated to expand CTLs in medium containing a combination of IL-1, IL-2, IL-4 and IL-6, followed by re-stimulation with the mitogen LPS, if it is possible to use lymph node lymphocytes isolated from lung cancer patients pre-injected with IL-2 as a CTL stimulant, the lymphocytes might be the best source to obtain antibody-producing hybridomas using lymphocytes activated by LPS.

In conclusion, lymph node lymphocytes exposed to LPS exhibited an Ab production efficiency at the clonal level following electrofusion with mouse myeloma cells which correlated strongly with the ratio of CD8⁺ to CD3⁺ cells present, while those exposed to SACI showed no significant correlations with the proportion of CD8⁺ cells present or with the ratio of CD8⁺ to CD3⁺ cells. These results suggest that the repertoire of B cells activated by LPS is different at least in part from that activated by SACI, and that the CD8/CD3 ratio could be used as a predictive parameter of Ab production efficiency following LPS stimulation.

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