Epstein-Barr Virus Transformation Induces B Lymphocytes to Produce Human Interleukin 10

By Nicolas Burdin, Catherine Péronne, Jacques Banchereau, and Françoise Rousset

From the Laboratory for Immunological Research, Schering-Plough, 69571 Dardilly, France

Summary

Interleukin 10 (IL-10) is a pleiotropic factor that enhances proliferation of activated human B lymphocytes and induces them to secrete high amounts of immunoglobulins. Here we show that several human B cell lines were able to constitutively secrete human (h)IL-10. Whereas none of the pre-B nor the plasmocytic cell lines tested produced hIL-10, 25 of the 36 tested mature B cell lines (lymphoblastoid and Burkitt lymphoma cell lines) secreted hIL-10. Moreover, 24 of these 25 hIL-10-producing B cell lines contained the Epstein-Barr virus (EBV) genome, suggesting a relationship between hIL-10 production by human B cell lines and EBV expression. Accordingly, whereas polyclonal activation via triggering of surface immunoglobulins or CD40 antigen induced highly purified normal human B lymphocytes to produce only low (0.3–0.4 ng/ml) but significant amounts of hIL-10, EBV infection induced them to secrete high amounts of hIL-10 (4–9 ng/ml). Furthermore, addition of exogenous hIL-10, simultaneously to EBV infection, potentiated cell proliferation, whereas a blocking anti-IL-10 antiserum inhibited it. Thus, hIL-10 produced by infected human B lymphocytes appears to be involved in the mechanisms of EBV-induced B cell proliferation.

To produce specific antibodies in response to antigenic stimulations (1), B lymphocytes interact with other immunocompetent cells. B cells are able to efficiently present antigen to T cells in an MHC-restricted context (2), and also to produce soluble immunomodulatory mediators. Indeed, both normal and malignant B lymphocytes have the ability to secrete cytokines. The constitutive or induced secretion of IL-1 (3), IL-6 (4), TNF (5, 6) and soluble CD23 (7) has already been reported. B lymphomas, myelomas, and EBV-infected B cell lines have been described to produce IL-1 (8), IL-5 (9), IL-6 (10), or TNF-α and -β (5, 6), with some of these cytokines suspected to act as autocrine growth factors (11–13).

IL-10 has been initially identified: (*a*) as a cytokine synthesis inhibitory factor, produced by Th2 cell clones, inhibiting macrophage-APC–dependent cytokine synthesis by Th1 cell clones (14); and (*b*) as a $Ly1^+$ B cell lymphoma product potentiating IL-2- or IL-4-dependent proliferation of thymocytes (15, 16). Human IL-10 (hIL-10)[1] and murine IL-10 (mIL-10) genes display >80% of homology (17, 18). Furthermore, hIL-10 exhibits a strong DNA and amino acid sequence homology to an open reading frame of the EBV genome: BCRF1 or viral IL-10 (vIL-10) (18). vIL-10 is expressed during the late stage of the virus cycle and exhibits most of the activities of h and mIL-10 (17–20). In the murine system, IL-10, just like IL-4, enhances MHC class II expression on resting B lymphocytes and sustains B cell viability in culture (21). In humans, IL-10 potentiates DNA replication of B cells stimulated via crosslinking of surface CD40 antigen or via surface Ig molecules (19). Moreover, IL-10 is a very potent differentiation factor and induces B cells to secrete high amounts of IgM, G, and A (19, 20).

The production of IL-10 by normal murine B cells has already been characterized at the mRNA level by PCR and at the protein level by ELISA (22). Moreover, as IL-10 is an efficient proliferation and differentiation factor for human B cells, the present study was initiated to determine whether human B lymphocytes were able to secrete this cytokine after polyclonal activation.

Materials and Methods

Reagents. Lymphoid B cell lines were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated FCS (MultiSer, Castel Hill, New South Wales, Australia), 2 mM glutamin (Gibco Laboratories), and 80 μg/ml of gentamycin (gentalline; Schering-Plough, Levallois-Perret, France). Cultures of normal B lymphocytes were carried out in modified Iscove's medium as detailed previously (23). Anti-IL-10 rat mAbs 12G8, 6B11, and 9D7 were kindly provided by Dr. J. Abrams (DNAX, Palo Alto, CA) (24) and used as purified products. The neutralizing

[1] *Abbreviations used in this paper:* CSIF, cytokine synthesis inhibitory factor; h, human; m, murine; SAC, *Staphylococcus aureus* Cowan I; v, viral.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/02/0295/10 $2.00
Volume 177 February 1993 295–304

rabbit polyclonal anti-IL-10 antibody was raised in the laboratory. Briefly, five subcutaneous and intramuscular injections of CHO-derived highly purified hIL-10 (100 μg to 1 mg of the soluble protein), in combination with CFA for the first injection and then IFA, were performed, every month, on female "Fauve de Bourgogne" rabbits. 1 wk after the fifth injection, blood was collected, and the antisera were isolated. The antisera were used at a 1:100 dilution (see below). The corresponding control was the preimmune serum of the same rabbits before hIL-10 was injected. The murine anti-CD40 mAb 89 (25) and anti-IFN-γ mAbs A35 and B27 (26) have been produced in our laboratory.

Insolubilized goat anti-human IgM antibody and formalinized particles of *Staphylococcus aureus* Cowan I (SAC) (Pansorbin) were purchased from Bio-Rad Laboratories (Richmond, CA) and from Calbiochem-Behring Corp. (La Jolla, CA), respectively. PMA and ionomycin were obtained from Calbiochem-Behring Corp., and PHA from Wellcome Laboratories (Paris, France). Cell phenotype was determined using FITC-conjugated mAbs originating from Becton Dickinson & Co. (Mountain View, CA) or Coulter Immunology (Hialeah, FL).

Purified rIL-2 (5×10^4 U/mg) was from Amgen Biologicals (Thousand Oaks, CA). The purified rIL-4 (10^7 U/mg; batch 8ILE1002) and hIL-10 (10^6 U/mg; batch 27785-82) were obtained, respectively, as *Escherichia coli* and CHO cell products from Schering-Plough Research Institute (Bloomfield, NJ). For the early experiments, the hIL-10 was produced in the laboratory using a baculovirus/insect cell system with a recombinant *Autographa californica* nuclear polyhedrosis virus genome containing the hIL-10 cDNA, kindly provided by Dr. K. W. Moore (DNAX). hIL-10 was used as *Spodoptera frugiperda* Sf9 cell supernatants after its infection by recombinant baculovirus. The yield was from 2 to 5 mg hIL-10 per liter of culture and was exactly calibrated against the purified rhIL-10. The vIL-10 used as standard in the vIL-10 ELISA was produced in the laboratory by Cos7 cells transfection with the vIL-10 cDNA-pF115 vector construct, also provided by Dr. K. W. Moore (DNAX). The Cos7 vIL-10 was calibrated against purified *E. coli*–derived recombinant vIL-10 (5×10^5 U/mg) provided by Dr. R. Kastelein (DNAX). In both cases, the mock control was performed using the empty vector.

Cell Lines. The CDw32/FcγRII-transfected Ltk^- cell line (CDw32 L cells) was described earlier (27). The Burkitt lymphoma BL, IARC 318, and IARC 518 cell lines were obtained from Dr. G. Lenoir (International Agency of Research on Cancer, Lyon, France). The majority of the lymphoblastoid cell lines has been obtained in our laboratory. The pre-B cell line pre-Alp was generated in the laboratory by Dr. D. Pandrau (manuscript in preparation). The chronic lymphocytic leukemia B cells were obtained from pathological samples, as described elsewhere (28). The other cell lines were from American Type Culture Collection (Rockville, MD).

Cell Preparations and Cell Cultures. B cells were isolated from tonsils, peripheral blood, spleen, and cord blood as previously described (29). The isolated population expressed >98% CD19 or CD20 (B cells), <1% CD2 (T cells), and <1% CD14 (monocytes). Isolation of $sIgD^+$ and $sIgD^-$ B cell populations was performed using a magnetic activated cell sorter as previously described (20). $CD5^+$ B lymphocytes were isolated by cell sorting on a FACStar Plus® (Becton Dickinson & Co.). When stimulated with SAC (0.05%), anti-μ (10 μg/ml), or PMA (1 ng/ml) + ionomycine (5 μg/ml), B cells were cultured at 10^6 cells/ml in 96-well flat-bottomed microtiter plates (Falcon Labware, Oxnard, CA). In the CD40 system (30, 31), purified B lymphocytes were cultured at $0.2–1 \times 10^6$ cells/ml with irradiated (7,500 rad) CDw32 L cells at $0.25–1 \times 10^5$ cells/ml and with 0.5 μg/ml of anti-CD40 mAb 89. IL-4 (100 U/ml), IL-2 (20 U/ml), and IL-10 (100 ng/ml) were added when appropriate. For EBV infection of purified B lymphocytes, 50 μl of a 100× concentrated culture supernatant of the B95.8 viral strain was added on B cells at 10^7/ml in RPMI. After 2 h of incubation at 37°C, cells were washed and cultured at 5×10^5 cells/ml. For each condition, cultures were performed in triplicates. Supernatants of these cultures were harvested at different time points and tested in the IL-10 ELISAs.

Cytokine Synthesis Inhibitory Factor (CSIF) Assay. The rabbit polyclonal anti-IL-10 antisera were tested for their ability to neutralize the activity of purified hIL-10 in CSIF assays, performed as previously described (14). Briefly, spleen mononuclear cells were activated in 96-well flat-bottomed microtiter plates (Falcon Labware), by a combination of SAC (0.05%), PHA (5 μg/ml), and IL-2 (100 U/ml) in the presence of serial concentrations of purified hIL-10 (0.1–100 ng/ml), with or without addition of anti-IL-10 or control antisera (diluted at 1:100, 1:1,000 or 1:10,000). The neutralizing effect of the anti-IL-10 antiserum on the inhibition of IFN-γ production induced by hIL-10 was determined after 72 h of culture using an IFN-γ ELISA as previously described (26).

IL-10 ELISAs. Both IL-10 ELISAs are developed on the pattern of a two-site sandwich ELISA format as previously described (26). One assay quantitates both h and vIL-10 while the other one is specific of vIL-10. Both assays rely on the same catcher mAb, 9D7, and the specificity is determined by the tracer biotinylated antibody: mAb 12G8 for the h + vIL-10 ELISA, and mAb 6B11 for the vIL-10 ELISA. Technically, the rat anti-IL-10 mAb, 9D7, was coated at 5 μg/ml for 2 h at 37°C, under 100 μl, in 96-well flat-bottomed microtiter plates (Nunc Immunoplate, Roskilde, Denmark). After 3 h of saturation with BSA, supernatants were incubated 2 h at room temperature. The standards consisted of CHO-derived purified hIL-10, with the concentration determined by amino acid analysis and Cos7-derived vIL-10, calibrated against purified rvIL-10. The tracer biotinylated mAbs were then incubated 2 h at room temperature: mAb 12G8 (v/hIL-10) was added at 0.05 μg/ml, and mAb 6B11 (vIL-10) was added at 0.1 μg/ml. The binding of the biotinylated antibodies was revealed with the alkaline phosphatase–conjugated streptavidin (Tago Inc., Burlingame, CA)/phosphatase substrate (Sigma Chemical Co., St. Louis, MO) system as previously described (26). The omission or the substitution with a nonrelated biotinylated mAb of any one of the mAbs completely switched off the signal. Furthermore, 10 μg/ml of hIL-10 did not react in the vIL-10 ELISA and the vIL-10 similarly reacted in both IL-10 ELISAs. The lower detection levels were 100 and 50 pg/ml for the h/vIL-10 and the vIL-10, respectively.

Results

Human B Cell Lines Constitutively Produce hIL-10. More than 40 malignant or EBV-transformed B cell lines were tested for their ability to produce IL-10. The results obtained for this screening are summarized in Table 1. 21 malignant cell lines were tested first. None of the five pro-B or pre-B cell lines produced IL-10. Among the 13 Burkitt lymphoma cell lines, representative of the different B cell maturation stages, five secreted hIL-10. With the exception of BJAB, all of these hIL-10-producing cell lines were EBV positive. However, four EBV-positive Burkitt lymphoma cell lines (BL29, BL60, BL110, and Daudi) failed to secrete hIL-10. The preplasmocytic cell line IARC 518, obtained from a patient suffering from a heavy chain disease, produced 1.7 ng/ml of hIL-10. Both plasmocytic cell lines U266 and RPMI 8866 did not

Table 1. *IL-10 Production by Human B Cell Lines*

Origin	B cell lines	EBV	hIL-10	vIL-10
			ng/ml	
Pro-B	Reh 6	–	<0.1	<0.05
Pre-B	Nalm 6, Pre-Alp, 697, IARC 318	–	<0.1	<0.05
Burkitt lymphoma	BL2, BL41, BL45, BL70	–	<0.1	<0.05
	BJAB	–	10.5 ± 3.5	<0.05
	BL29, BL60, BL110, Daudi	+	<0.1	<0.05
	BL16	+	3.1 ± 0.5	<0.05
	BL18	+	1.3 ± 0.2	<0.05
	BL74	+	0.9 ± 0.3	<0.05
	Jijoye	+	6.8 ± 2	<0.05
Lymphoblastoid	IM9, RPMI8866, UD195	+	<0.1	<0.05
	16 different lymphoblastoid cell lines	+	0.3–11	<0.05
	UD189	+	11.2 ± 3	0.2 ± 0.05
	UD194	+	3.7 ± 1.3	0.2 ± 0.1
	L38	+	3 ± 1.2	0.2 ± 0.04
	C11	+	5 ± 1	0.3 ± 0.1
Heavy chain disease	IARC 518	–	1.7 ± 0.5	<0.05
Myeloma	U266, RPMI 8226	–	<0.1	<0.05

The cell line supernatants were harvested after 48–96 h of culture and then tested in the IL-10 ELISAs. For each B cell line, IL-10 concentrations are expressed as mean ± SD of two to five independent assays.

secrete hIL-10. 23 lymphoblastoid B cell lines, established from peripheral blood of normal donors, were then screened. 20 of them secreted 0.3–11 ng/ml of hIL-10.

Therefore, mature human B cell lines were able to produce hIL-10, and this secretion appeared to be at least partially correlated with EBV expression since 24 of the 26 hIL-10-secreting cells were EBV positive. Besides, it should be stressed that only low levels of vIL-10 could be detected in the supernatants of a restricted number of EBV-infected cell lines.

The kinetic of hIL-10 secretion by a human B cell line was then investigated using the EBV-positive Burkitt lymphoma cell line Jijoye. B cells were initially seeded at 5×10^4 cells/ml, and the viable cell recovery was determined daily over a 96-h kinetic. The corresponding supernatants were harvested and assayed in the IL-10 ELISAs. As illustrated in Fig. 1, the hIL-10 levels detected in the supernatants increased together with cell concentration: from 0.2 ng/ml for 10^5 cells/ml after 24 h of culture, to 8.1 ng/ml for 1.4×10^6 cells/ml after 96 h of culture. The hIL-10 levels did not change even when cultures were left unsplit for several more days, resulting in a strong cell death. Thus, the highest hIL-10 concentrations were found in the highest density Jijoye cell cultures.

Normal Human B Lymphocytes Secrete Low Levels of hIL-10 upon Polyclonal Activation. To determine whether normal human B cells could also produce IL-10, highly purified B cells and total mononuclear cells, as control, were stimulated either with polyclonal activators such as PMA plus ionomycine, SAC, or anti-IgM, or in the CD40 system. Addition of IL-2 or IL-4 and combinations of two different activation

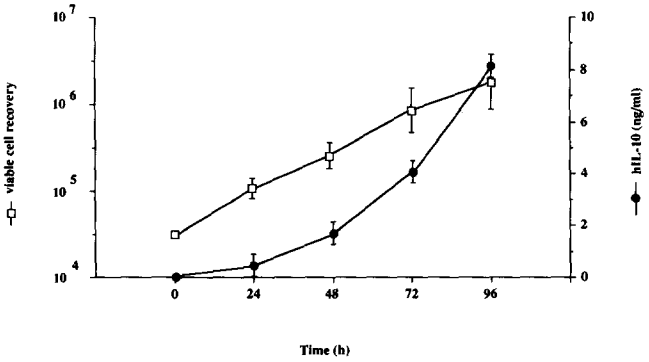

Figure 1. Kinetic of hIL-10 production by a lymphoid cell line. The EBV-positive Burkitt lymphoma cell line Jijoye was cultured at the initial cell concentration of 5×10^4 cells/ml. Viable cell recovery (□) was determined by trypan blue dye exclusion, and supernatants were assayed in the IL-10 ELISA (●) every 24 h on a 96-h kinetic.

Table 2. *IL-10 Production by Tonsillar Normal Human Lymphoid Cells*

Cells	Activator	hIL-10	vIL-10	Time
		ng/ml	*ng/ml*	*h*
Mononuclear cells				
(10^6 cells/ml)	None*	<0.1	<0.05	-
	SAC + PHA + IL-2*	4.02 ± 0.07	<0.05	72
Purified B cells				
(10^6 cells/ml)	None*	<0.1	<0.05	-
	PMA + ionomycine‡	0.36 ± 0.1	<0.05	72
	SAC*	0.34 ± 0.06	<0.05	48
	SAC + IL-2‡	0.30 ± 0.03	<0.05	48
	Anti-μ*	0.36 ± 0.05	<0.05	48
	Anti-μ + IL-2‡	0.35 ± 0.09	<0.05	48
	CD40*	0.39 ± 0.02	<0.05	24
	CD40 + IL-4*	0.34 ± 0.07	<0.05	24
	CD40 + IL-2‡	0.40 ± 0.09	<0.05	48
	CD40 + SAC*	0.32 ± 0.08	<0.05	24
	CD40 + SAC + IL-2‡	0.37 ± 0.06	<0.05	48
	CD40 + anti-μ‡	0.38 ± 0.1	<0.05	48
	CD40 + anti-μ + IL-2‡	0.34 ± 0.05	<0.05	48
Purified B cells				
(2×10^6 cells/ml)	None‡	<0.1	<0.05	-
	CD40 + SAC‡	0.74 ± 0.05	<0.05	24

Mononuclear cells or purified B cells were cultured at 10^6 or 2×10^6 cells/ml. SAC was used at 0.05%, anti-μ at 10 μg/ml, PMA at 1 ng/ml, ionomycine at 5 μg/ml, PHA at 5 μg/ml, IL-4 at 100 U/ml, and IL-2 at 20 U/ml. For the CD40 system reported as CD40, mAb 89 was used at 0.5 μg/ml, and CDw32-transfected L cells at 0.5×10^5 cells/ml. For a given culture condition, the values of IL-10 production shown represent the highest level detected during a 10-d kinetic. The time of culture for which this level was obtained, reproducible for a given culture condition, is also mentioned in each case. Results are expressed as mean ± SD of independent experiments, all done in triplicates cultures.
* Four experiments.
‡ Two experiments.

procedures have also been tested. The efficiency of the activation was checked by [^{3}H]thymidine uptake or by Ig production measurements. B cell culture supernatants were harvested at different time points: from 4 h to 10 d, and then tested in the h/v and vIL-10 ELISAs. The results illustrated in Table 2 were pooled from several independent experiments performed with tonsillar purified B lymphocytes cultured under the same conditions. Nonactivated B cells did not produce IL-10. Upon activation, purified B cells secreted low (compared with activated total mononuclear cells) but significant and reproducible levels of hIL-10, but no vIL-10. For each activation mode, the hIL-10 concentration increased up to 0.3–0.4 ng/ml after 24–72 h and then progressively decreased down to the detection limit level during the next 2–3 d (see a typical kinetic in Fig. 2). As shown in Table 2, doubling the number of B cells at culture onset resulted in the secretion of twice as much hIL-10. The activation of total tonsillar mononuclear cells in the CD40 system, with or without IL-4, did not lead

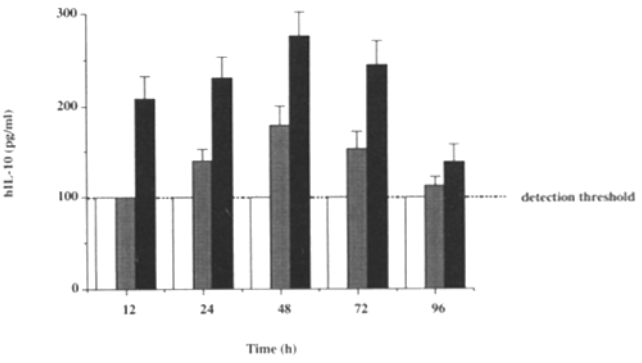

Figure 2. hIL-10 production by CD5-enriched human B cells. CD5-enriched B cells (<1.5% CD2$^+$, <4% CD14$^+$, >80% CD5$^+$) isolated from cord blood were cultured in 10^6 cells/ml in medium alone (□), with SAC + IL-2 (▨), or in the CD40 system + SAC (■). In each case, the production of hIL-10 on a 96-h kinetic was assayed by IL-10 ELISAs. No production of vIL-10 was detected. Results are expressed as mean ± SD of triplicate cultures.

to higher hIL-10 concentrations (data not shown), suggesting that, upon these B-specific activation procedures, the weak percentage of contaminant non-B cells could not be responsible for the hIL-10 levels observed in Table 2. Similar levels and kinetics of hIL-10 production were also obtained with human B cells isolated from spleen and peripheral blood (data not shown).

We then investigated whether hIL-10 production was restricted to a subpopulation of normal human B cells. First, supernatants of activated IgD^+ and IgD^- B lymphocytes contained levels of hIL-10 comparable to those of the unseparated population (data not shown). Second, $CD5^+$ human B cells purified from cord blood were tested for their ability to secrete hIL-10 either spontaneously or after activation under conditions resulting in the production of high Ig levels, such as SAC plus IL-2 or the combination of SAC with the CD40 system (20). As shown in Fig. 2, $CD5^+$ B lymphocytes did not constitutively secrete hIL-10. Stimulated $CD5^+$ B cells were able to produce low levels of hIL-10. The secretion was observed after 12 h and the peak was reached after 48 h of culture. Otherwise, resting or activated chronic lymphocytic leukemia $CD5^+$ B cells from five different patients tested did not secrete IL-10 (data not shown). Thus, in contrast to the results generated in the murine system (22, 32), the $CD5^+$ B lymphocytes subpopulation did not appear to constitute the main source of hIL-10 production within the whole human B cell population.

Taken together, these results indicate that activated normal human B lymphocytes are able to secrete significant levels of hIL-10, but did not reach those obtained for human B cell lines.

Normal Human B Lymphocytes Secrete High Amounts of hIL-10 after EBV Infection. Since most of the EBV-positive B cell lines have the ability to produce hIL-10, we wondered whether EBV infection of normal B lymphocytes would induce them to secrete IL-10 levels higher than those observed in response to the polyclonal activators. Thus, tonsillar purified B cells were infected by EBV and cultured for 30 d. At different time points, culture supernatants were harvested and tested in the IL-10 ELISAs and viable cell recovery was determined using trypan blue dye exclusion. For the experiment illustrated in Fig. 3, medium changes, indicated by arrows, were performed when B cell concentration reached saturation at $\sim 3 \times 10^6$ cells/ml. During the first 6 d after EBV infection, when strong cell death occurred, no significant amount of IL-10 could be detected in culture supernatants. However, an induction of hIL-10 production was observed in supernatants 6 d after the culture onset, when the number of initially seeded cells was recovered. Thereafter, hIL-10 concentrations increased up to 9 ng/ml at day 14, when all the B lymphocytes acquired the typical morphology of immortalized lymphoblastoid cells and expressed Epstein-Barr nuclear antigens as detected by immunofluorescence (data not shown). At later time points, cells continued to grow with a high proliferation rate, while the hIL-10 level slowly decreased down to ~1 ng/ml, where it remained stable for >6 wk (data not shown). The EBV infection of purified tonsillar B cells isolated from 12 different donors led to the same results with on average 6.6 ± 1.8 ng/ml of hIL-10 detected at the peak, obtained between days 14 and 19. Interestingly, no vIL-10 could be detected at any time of the kinetics. Thus, EBV transformation induces purified B lymphocytes to secrete high amounts of hIL-10.

Addition of Exogenous IL-10 Potentiates EBV-induced B Cell Proliferation. To examine further the role of hIL-10 during EBV infection of normal B cells, the effect of exogenous hIL-10

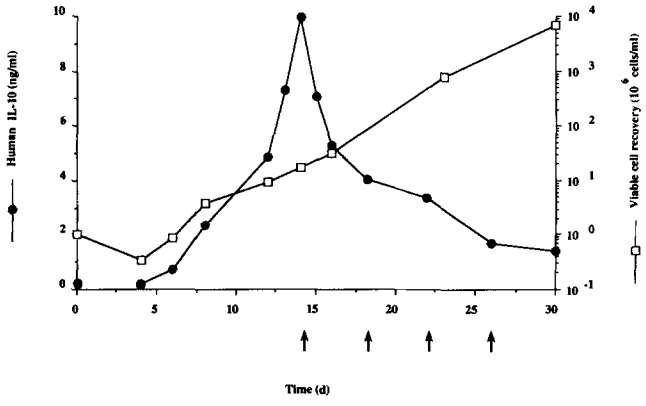

Figure 3. hIL-10 production during EBV transformation of purified human B cells. Tonsillar purified B lymphocytes were infected by EBV as described in Materials and Methods and then cultured at 5×10^5 cells/ml. Supernatants were harvested at different time points and then assayed in the IL-10 ELISAs. Viable cell recovery (□) was determined by trypan blue dye exclusion at different time points after EBV infection. Cell cultures were split at one fourth at the time points indicated by arrows. EBV transformation of purified B lymphocytes induced hIL-10 secretion (●). No vIL-10 induction was detected at any time of the kinetic.

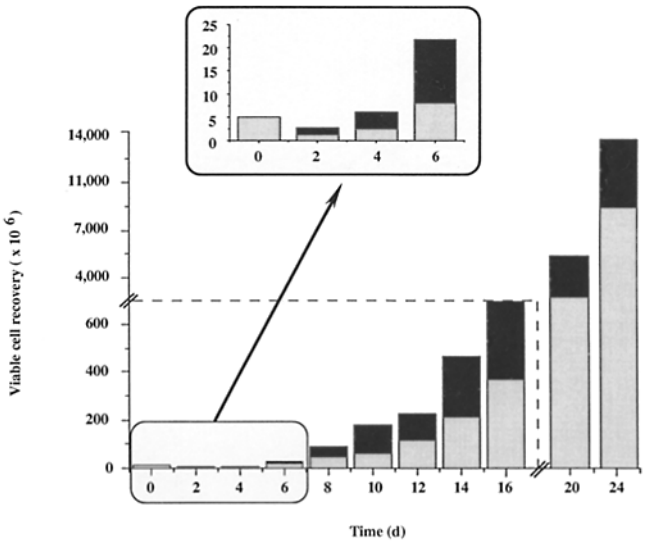

Figure 4. Exogenous hIL-10 potentiates proliferation of EBV-infected B lymphocytes. EBV-infected tonsillar B lymphocytes were cultured at 5×10^5 cells/ml without (▩) or with (■) hIL-10 (100 ng/ml), added only at day 0. During the 24 d after infection, the cell number was determined using trypan blue dye exclusion. Identical medium changes occurred for both conditions (with or without hIL-10) at the same time points.

on cell growth was tested. Purified rhIL-10 was added at day 0, immediately after purified B lymphocytes had been incubated 2 h with EBV. The number of viable B cells was determined at different time points over a 20-d kinetic. Results shown in Fig. 4 are expressed as cumulative theoretical cell numbers. The addition of exogenous hIL-10 accelerated the recovery of the viable cell number initially seeded (day 4 with exogenous hIL-10 vs. day 6 for the control medium). This enhancement of cell proliferation was quite potent between days 7 and 16, when addition of exogenous hIL-10 induced a twofold increase of viable B cells. Thereafter, the effect of exogenous hIL-10 became less pronounced. This potentiating effect of exogenous hIL-10 was observed on five independent experiments performed with purified B cells isolated from different donors. These results suggested that hIL-10 was a proliferation factor for EBV-infected B cells. Therefore, the endogenous secretion of hIL-10 observed during EBV infection of purified B lymphocytes could be involved in the mechanisms sustaining cell proliferation and leading to the establishment of the cell line.

Anti-IL-10-neutralizing Antiserum Inhibits EBV-induced B Cell Proliferation. To further study the role of endogenously produced hIL-10 in the proliferation of EBV-infected B cells, we generated neutralizing polyclonal anti-IL-10 antibodies. Rabbits were immunized with highly purified rhIL-10 as described in Materials and Methods. The antisera obtained after five injections were tested for their ability to antagonize the blocking effect of purified rhIL-10 on the IFN-γ production by activated spleen mononuclear cells. As shown in Fig. 5, the anti-IL-10 antiserum, diluted at 1:100, completely blocked the effect of 10 ng/ml of hIL-10 and antagonized 80% of the effect of 100 ng/ml, while the preimmune control serum remained inactive.

So as to block endogenous hIL-10, the neutralizing or control antisera, diluted at 1:100, were added at the onset of the culture, immediately after EBV infection of B cells and thereafter every 5 d. The viable cell recovery was determined for each culture condition at different time points of the kinetic. Fig. 6 illustrates the results obtained for one of three independent experiments leading to the same following observations. The addition of anti-IL-10 antiserum delayed the recovery of the initial cell number and strongly reduced the cell proliferation over the 22-d culture. At day 17, the number of viable cells cultured with anti-IL-10 antiserum was fivefold lower than that of cells cultured with control antiserum or in medium alone. During the same period, the control serum remained inactive. Experiments, where increasing dilutions (1:100, 1:500, 1:1,000, 1:5,000) of the anti-IL-10 and control antisera were added to infected B cells, were also performed. As illustrated in Fig. 7 A, the inhibition of cell growth was dose dependent and already observed with a dilution of 1:1,000 of the anti-IL-10 antiserum. Whatever the dilutions, the control antiserum remained inactive. Moreover, Fig. 7 *A* also illustrates that addition of 250 ng/ml of exogenous hIL-10 to the 1:100 dilution of the neutralizing antiserum completely reversed its inhibitory effect and even resulted in the potentiation of B cell growth described above.

To determine when the anti-IL-10 antiserum preferentially inhibited cell growth during the 22-d kinetic shown in Fig. 6, proliferation rates, obtained without or with anti-IL-10 or control antisera, were calculated for two intervals: between days 0 and 17, and between days 17 and 22. As illustrated in Fig. 7 *B*, the inhibition of the proliferation rate mediated by the neutralizing anti-IL-10 antiserum reached 80% during the first time interval, whereas the proliferation rates were identical after 17 d. Moreover, when studied after 10 wk, the cell lines established either with medium alone, control antiserum, or anti-IL-10 antiserum grew with approximately the same proliferation rate (data not shown). This indicates

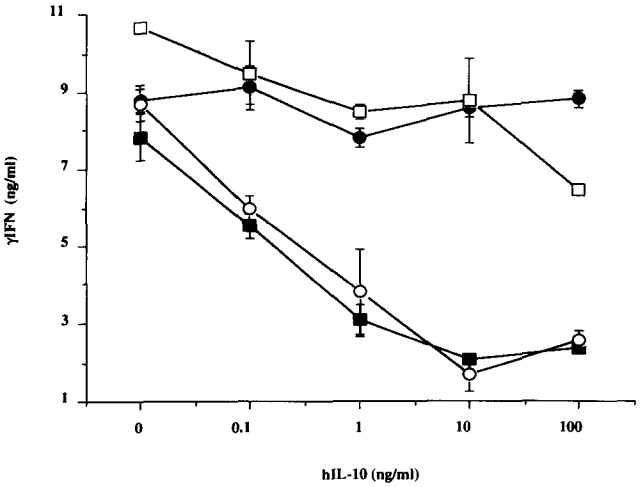

Figure 5. The rabbit polyclonal anti-IL-10 antiserum neutralizes the effect of pure hIL-10 in a CSIF assay. Spleen mononuclear cells were activated as described in Materials and Methods. The initial IFN-γ level (●) was dose-dependently inhibited by addition of increasing concentrations of pure hIL-10 (■). The anti-IL-10 (□) or control (○) antisera diluted at 1:100 were assayed for their ability to neutralize this inhibitory effect.

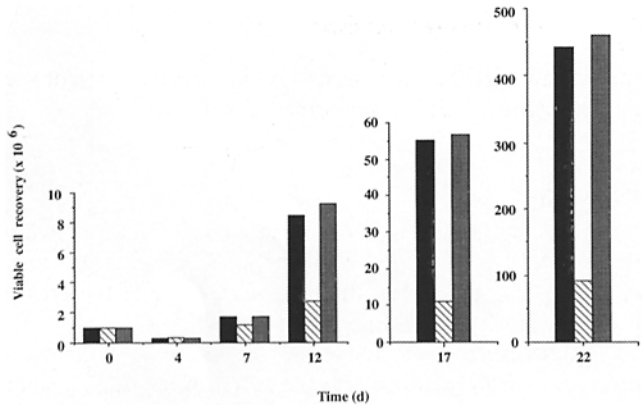

Figure 6. A blocking polyclonal anti-IL-10 antiserum strongly reduces proliferation of EBV-transformed B cells. EBV-infected tonsillar B lymphocytes were cultured at 5 × 10^5 cells/ml without (■) or with anti-IL-10 antiserum (▨) or control serum (▩) diluted at 1:100. The number of viable cells was determined on a 22-d kinetic by trypan blue dye exclusion. Medium changes were performed at the same time points for each culture condition. Cells were reseeded at 2.5 × 10^5 cells/ml.

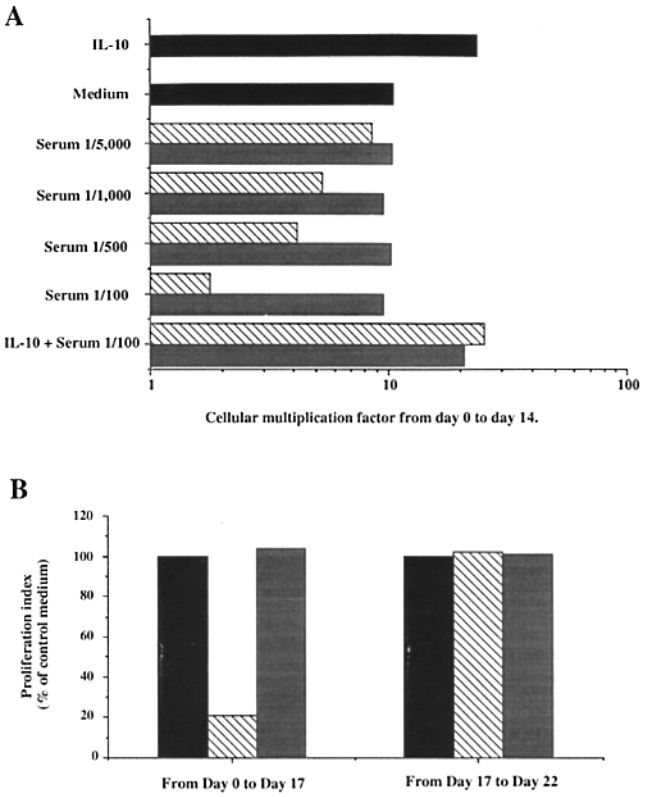

Figure 7. The effect of a blocking polyclonal anti-IL-10 antiserum is dose dependent and reversible but does not prevent establishment of the cell line. EBV-infected tonsillar B lymphocytes were cultured at 5×10^5 cells/ml without (■) or with anti-IL-10 antiserum (▨) or control serum (▩). (*A*) A dose-dependent experiment was performed with different dilutions of the anti-IL-10 antiserum and control serum (1:100, 1:500, 1:1,000, 1:5,000), and the reversibility of this effect was tested by addition of exogenous hIL-10 (250 ng/ml) combined with the sera at 1:100. (*B*) The proliferation index (which represented the ratio of the proliferation rate of the condition anti-IL-10 or control antiserum over the proliferation rate of the condition medium alone) was determined for two time intervals of the experiment illustrated in Fig. 6: between days 0 and 17, and between days 17 and 22. Results are expressed as percentage of control medium.

that the anti-IL-10 antiserum delays but does not prevent the establishment of EBV-transformed B cell lines.

Discussion

The screening of >40 B cell lines suggests that the secretion of IL-10 is associated with the mature and preplasmocytic stages of B cell development, since none of the pro-B, pre-B, or myeloma cell lines tested were able to produce IL-10. The data also indicate an association between the presence of the EBV genome and the secretion of hIL-10, since 24 of 31 EBV-positive mature B cell lines secrete IL-10, whereas only one of five EBV-negative mature B cell lines did so. It now remains to establish whether viral proteins such as EBNA 2-6 or latent membrane protein may be involved in the transactivation of the hIL-10 gene, as it has already been shown for several B cell activation markers such as CD23 or Bac-1 (33). Nonetheless, a few EBV-positive B cell lines do not produce hIL-10, and two EBV-negative B cell lines were able to secrete hIL-10 (BJAB and IARC 518). BJAB may produce hIL-10 as a consequence of an intricate pattern of chromosomic translocations, different from the classical translocations that exist in other Burkitt lymphomas (34). The mechanisms leading to the production of hIL-10 by the preplasmocytic cell line IARC 518 also remain to be elucidated. This strong association between EBV expression and hIL-10 production by a human B cell line is consistent with the results recently obtained with human B cell lines derived from patients with AIDS and Burkitt lymphoma (35).

It is important to emphasize that vIL-10 was only seldomly detected and, if so, at low levels. In fact, vIL-10 has been described as being expressed only during the late phase of the virus lytic cycle (36). Thus, the occasional detection of only low amounts of vIL-10 in the supernatants of lymphoblastoid cell lines is consistent with the fact that EBV is present in these cells in a latent state. It will be of interest to check whether the cell lines producing vIL-10 are also able to produce the virus. Finally, the fact that EBV induces the expression of a cellular gene (hIL-10), with a viral counterpart (vIL-10) already existing within the viral genome, still remains an unresolved issue.

The present study also indicates that the production of hIL-10 by human B cells is not restricted to cell lines since normal B lymphocytes can also secrete hIL-10 after activation. These results are consistent with the detection by PCR of the hIL-10 mRNA for some EBV-transformed B cell lines and also for SAC-activated normal human B lymphocytes (18). However, whatever the B cell origin (tonsils, spleen, peripheral blood, or cord blood) and the polyclonal activator used, the maximum of hIL-10 production was low (0.35 ng/ml for 10^6 cells initially seeded). In fact, these levels are comparable to those described for mouse B cells (22). None of the tested B cell subpopulations (tonsil IgD^+ or IgD^-, cord blood $CD5^+$) was found to produce higher levels of hIL-10. This is in apparent contrast to results obtained with mouse B cells, where IL-10 production appears to be associated with CD5 expression (32).

The biological meaning of such a level of IL-10 secretion remains difficult to establish as concentrations of hIL-10 <0.5 ng/ml do not affect B cell growth or differentiation (19). Thus, this endogenous production of hIL-10 by B cells does not seem to be sufficient to induce autocrine B cell proliferation or differentiation. This is consistent with the strong B cell progression activity of exogenous IL-10 observed in the CD40 system (19, 20). In this context, we were particularly surprised by the low hIL-10 levels observed in response to the combination of the CD40 system and SAC, conditions that represent a thymoindependent B cell activation pathway, where large amounts of Igs are secreted (20). Indeed, we had hypothesized that the important Ig production observed under these culture conditions was the consequence of a high production of endogenous hIL-10. However, an autocrine loop, occurring without release of IL-10 outside cells, might happen then.

In our hands, EBV was the sole agent able to induce normal human B lymphocytes to secrete high levels of IL-10. Indeed, after EBV transformation of purified normal B cells, a notice-

able production of hIL-10 was observed, whereas no vIL-10 induction was detectable. The hIL-10 secretion was well correlated with the growth of infected cells. The peak of production extended from day 12 to 18, a critical stage for establishment of the cell line. Moreover, two findings indicate that this production of hIL-10 plays a facilitating role in the establishment of the transformed cell line. First, addition of exogenous hIL-10 at the onset of the culture resulted in an earlier and stronger proliferation of the transformed cells. Second, the addition of a blocking polyclonal anti-IL-10 antiserum efficiently slowed down cell growth. However, the neutralizing antiserum did not prevent the transformation of B lymphocytes and the establishment of the cell line. This is consistent with the results obtained with antiviral cytokines as powerful as IFN-α or IFN-γ, which delay but do not prevent the establishment of EBV-transformed cell lines (37, 38). Taken together, these results suggest that the endogenous hIL-10 secretion is closely associated to the EBV-induced B cell proliferation during the early stages of cell transformation. Once established, the cell line apparently becomes independent of hIL-10 since exogenous hIL-10 or anti-IL-10 antibody turn out to be inefficient at this stage. In this context, several cytokines have already been proposed as autocrine growth factors for long-term established human lymphoblastoid cell lines: IL-5 (39), IL-6 (8), adult T cell leukemia–derived factor/thioredoxin (40), TNF-β (9), or soluble CD23 (10). We are presently trying to determine whether hIL-10 plays an autocrine role in the proliferation of Burkitt lymphoma B cell lines and normal B lymphocytes. This may be difficult to establish since the growth of B cells is likely to depend on the coordinated action of several redundant growth factors.

Since secretion of hIL-10 appears in vitro to be related to the proliferation of EBV-infected B lymphocytes, it is tempting to speculate that this cytokine may be involved in vivo in EBV-associated B cell lymphoproliferative disorders. These include infectious mononucleosis and EBV-associated B cell lymphomas of immunocompromised subjects. Studies are presently under way to determine whether patients suffering from such diseases exhibit abnormal levels of hIL-10 within the tumoral area or in their biological fluids. In addition, this endogenous production of hIL-10 by EBV-transformed B lymphocytes may also be involved in an immunosuppressive process in response to the viral infection through two different mechanisms: by inhibiting the production of IFN-γ, a potent antiviral cytokine (38) produced by T cells and NK cells (14), and by inhibiting antigen presentation to T cells (41). Thus, EBV may induce the secretion of IL-10 by B lymphocytes to protect itself from the immune reaction subsequent to viral infection. In this context, we recently found that simultaneous addition of EBV and IL-10 on total PBMC from EBV-positive donors suppresses reactivation of EBV-specific cytotoxicity and permits establishment of lymphoblastoid cell lines (Burdin, N., and F. Rousset, unpublished observations). Taken together, these results suggest that the IL-10 production by EBV-transformed B cells may also represent an evasion mechanism against the host antiviral systems. Therefore, the EBV-induced production of hIL-10 may play a dual role: (*a*) by suppressing the antiviral arms of the immune system, and (*b*) by enhancing the survival and the growth of their hosts, the B lymphocytes.

We express our gratitude to Dr. J. Abrams (DNAX, Palo Alto, CA) for the kind gift of the anti-IL-10 mAbs. We thank Dr. G. Lenoir and Dr. A. Calender (IARC) for stimulating discussions, and L. Galibert and Dr. F. Brière for many helpful discussions and careful reading of the manuscript. We are grateful to Drs. D. Pandrau and A. C. Fluckiger for providing the Pre-Alp cell line and B-CLL samples, respectively. We also thank Dr. J. Chiller for critical review of the manuscript.

N. Burdin is the recipient of a grant from the Fondation Mérieux (Lyon, France).

Address correspondence to Nicolas Burdin, Laboratory for Immunological Research, Schering-Plough, 27, Chemin des Peupliers, B.P. 11, 69571, Dardilly Cédex, France.

Received for publication 17 August 1992 and in revised form 28 October 1992.

Note added in proof: During the printing of our manuscript, supporting data have been reported, indicating that IL-10 is produced by malignant cells in AIDS B cell lymphomas associated with EBV, as detected by in situ hybridization (42).

References

1. Banchereau, J., and F. Rousset. 1992. Human B lymphocytes: phenotype, proliferation and differentiation. *Adv. Immunol.* 52:125.
2. Metlay, J.P., E. Puré, and R.M. Steinman. 1989. Control of the immune response at the level of antigen-presenting cells: a comparison of the function of dendritic cells and B lymphocytes. *Adv. Immunol.* 47:45.
3. Bonnefoy, J.Y., M.C. Denoroy, O. Guillot, C.L. Martens, and J. Banchereau. 1989. Activation of normal human B cells through their antigen receptor induces membrane expression of IL-1α and secretion of IL-1β. *J. Immunol.* 143:864.
4. Smeland, E.B., H.K. Blomhoff, S. Funderud, M.R. Shalaby,

and T. Espevik. 1989. Interleukin 4 induces selective production of interleukin 6 from normal human B lymphocytes. *J. Exp. Med.* 170:1463.
5. Sung, S.S.J., L.K.L. Jung, J.A. Walters, W. Chen, C.I. Wang, and S.M. Fu. 1988. Production of tumor necrosis factor/cachectin by human B cell lines and tonsillar B cells. *J. Exp. Med.* 168:1539.
6. Sung, S.S.J., L.K.L. Jung, J.A. Walters, G.A. Granger, and S.M. Fu. 1989. Production of lymphotoxin by isolated human tonsillar B lymphocytes and B lymphocytes cell lines. *J. Clin. Invest.* 84:236.
7. Cairns, J., L. Flores-Romo, M.J. Millsum, G.R. Guy, S. Gillis, J.A. Ledbetter, and J. Gordon. 1988. Soluble CD23 is released by B lymphocytes cycling in response to IL-4 and anti-Bp50 (CDw40). *Eur. J. Immunol.* 18:349.
8. Pistoia, V., F. Cozzolino, A. Rubartelli, M. Torcia, S. Roncella, and M. Ferrarini. 1986. In vitro production of IL-1 by normal and malignant human B lymphocytes. *J. Immunol.* 136:1688.
9. Paul, C.C., J.R. Keller, J.M. Armpriester, and M.A. Baumann. 1990. Epstein-Barr virus transformed B lymphocytes produce IL-5. *Blood.* 75:1400.
10. Hutchins, D., B.B. Cohen, and C.M. Steel. 1990. Production and regulation of IL-6 in human B lymphoid cells. *Eur. J. Immunol.* 20:961.
11. Yokoi, T., T. Miyawaki, Y.K. Kato, Y. Kashara, and N. Taniguchi. 1990. Epstein-Barr-virus-immortalized B cells produce IL-6 as an autocrine growth factor. *Immunology.* 70:100.
12. Seregina, T.M., M.I. Mekshenkov, R.L. Turetskaya, and S.A. Nedospasov. 1989. An autocrine growth factor constitutively produced by a human lymphoblastoid B-cell line is serologically related to lymphotoxin (TNF β). *Mol. Immunol.* 26:339.
13. Gordon, J. 1990. Autocrine aspects of the B lymphocyte response. *In* Cytokines and B lymphocytes. R.E. Callard, editor. Academic Press Limited, London. 195–213.
14. Fiorentino, D.F., M.W. Bond, and T.R. Mosmann. 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* 170:2081.
15. Suda, T., A. O'Garra, I. MacNeil, M. Fischer, M. Bond, and A. Zlotnik. 1990. Identification of a novel thymocyte growth promoting factor derived from B cell lymphomas. *Cell. Immunol.* 129:228.
16. MacNeil, I., T. Suda, K.W. Moore, T.R. Mosmann, and A. Zlotnik. 1990. IL-10: a novel cytokine growth cofactor for mature and immature T cells. *J. Immunol.* 145:4167.
17. Moore, K.W., P. Vieira, D.F. Fiorentino, M.L. Trounstine, T.A. Khan, and T.R. Mosmann. 1990. Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRF1. *Science (Wash. DC).* 248:1230.
18. Vieira, P., M.N. Dang, R. Kastellein, D.F. Fiorentino, J.E. de Vries, M.G. Roncarolo, T.R. Mosmann, and K.W. Moore. 1991. Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRF1. *Proc. Natl. Acad. Sci. USA.* 88:1172.
19. Rousset, F., E. Garcia, T. Defrance, C. Péronne, N. Vezzio, D. Hsu, R. Kastelein, K.W. Moore, and J. Banchereau. 1991. Human and viral IL-10 are potent growth and differentiation factors for activated human B lymphocytes. *Proc. Natl. Acad. Sci. USA.* 89:1890.
20. Defrance, T., B. Vanbervliet, F. Brière, I. Durand, F. Rousset, and J. Banchereau. 1991. Viral IL-10 and TGFβ cooperate to induce anti-CD40-activated naive human B cells to secrete IgA. *J. Exp. Med.* 175:671.
21. Go, N.F., B.E. Castle, R. Barett, R. Kastelein, W. Dang, T.R. Mosmann, K.W. Moore, and M. Howard. 1990. Interleukin 10, a novel B cell stimulatory factor: unresponsiveness of X chromosome-linked immunodeficiency B cells. *J. Exp. Med.* 172:1625.
22. O'Garra, A., G. Stapelton, V. Dhar, M. Pearce, H. Rugo, D. Barbis, J. Cupp, K. Moore, P. Vieira, T. Mosmann, L. Arnold, G. Haughton, and M. Howard. 1990. Production of cytokines by mouse B cells: B lymphomas and normal B cells produce interleukin 10. *Int. Immunol.* 2:821.
23. Yssel, H., J.E. de Vries, M. Kuren, and M. Spits. 1984. Serum free medium for generation and propagation of functional human cytotoxic and helper T cell clones. *J. Immunol. Methods.* 72:219.
24. Abrams, J.S., M.G. Roncarolo, H. Yssel, U. Anderson, G.J. Gleich, and J.E. Silver. 1992. Strategies of anti-cytokine monoclonal antibody development: immunoassay of IL-10 and IL-5 in clinical samples. *Immunol. Rev.* 127:5.
25. Vallé, A., C.E. Zuber, T. Defrance, O. Djossou, M. De Rie, and J. Banchereau. 1989. Activation of human B lymphocytes through CD40 and interleukin-4. *Eur. J. Immunol.* 19:1463.
26. Favre, C., J. Wijdenes, H. Cabrillat, O. Djossou, J. Banchereau, and J.E. de Vries. 1989. Epitope mapping of recombinant human gamma-interferon using monoclonal antibodies. *Mol. Immunol.* 26:17.
27. Peltz, G.A., M.L. Trountsine, and K.W. Moore. 1988. Cloned and expressed human Fc receptor for IgG mediates anti-CD3 dependent lymphoproliferation. *J. Immunol.* 141:1891.
28. Fluckiger, A.C., J.F. Rossi, A. Bussel, P. Bryon, J. Banchereau, and T. Defrance. Responsiveness of chronic lymphocytic leukemia B cells activated via surface Igs or CD40 to B cell tropic factor. *Blood.* In Press.
29. Defrance, T., B. Vanbervliet, J.P. Aubry, Y. Takabe, N. Arai, A. Miyajima, T. Yokota, F. Lee, K. Arai, J.E. de Vries, and J. Banchereau. 1987. B cell growth promoting activity of recombinant human Interleukin-4. *J. Immunol.* 139:1135.
30. Banchereau, J., P. de Paoli, A. Vallé, E. Garcia, and F. Rousset. 1991. Long term human B cell lines dependent on interleukin-4 and antibody to CD40. *Science (Wash. DC).* 251:70.
31. Rousset, F., E. Garcia, and J. Banchereau. 1991. Cytokine-induced proliferation and immunoglobulin production of human B lymphocytes triggered through their CD40 antigen. *J. Exp. Med.* 173:705.
32. O'Garra, A., R. Chang, N. Go, R. Hastings, G. Haughton, and M. Howard. 1992. Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. *Eur. J. Immunol.* 22:711.
33. Calender, A., M. Billaud, J.P. Aubry, J. Banchereau, M. Vuillaume, and G.M. Lenoir. 1987. Epstein-Barr Virus (EBV) induces expression of B cell activation markers on *in vitro* infection of EBV-negative B lymphoma cells. *Proc. Natl. Acad. Sci. USA.* 84:8060.
34. Lenoir, G.M., and G.W. Bornkamm. 1987. Burkitt's lymphoma, a human cancer model for the study of multistep development of cancer: proposal for a new scenario. *Adv. Viral Oncol.* 7:173.
35. Benjamin, D., T.J. Knobloch, and M.A. Dayton. 1992. Human B cell interleukin-10: B cell lines derived from patients with acquired immunodeficiency syndrome and Burkitt's lymphoma constitutively secrete large quantities of interleukin-10. *Blood.* 80:1289.
36. Hudson, G.S., A.T. Bankier, S.C. Satchwell, and B.G. Et Barell. 1985. The short unique region of the B95.8 Epstein-Barr virus genome. *Virology.* 147:81.

37. Garner, J.G., M.S. Hirsch, and R.T. Schooley. 1984. Prevention of Epstein-Barr virus-induced B cell outgrowth by interferon alpha. *Infect. Immun.* 43:920.
38. Lotz, M., C.D. Tsoukas, S. Fong, D.A. Carson, and J.H. Vaughan. 1985. Regulation of Epstein-Barr virus infection by recombinant interferons. Selected sensitivity to interferon gamma. *Eur. J. Immunol.* 15:520.
39. Baumann, M.A., and C.C. Paul. 1992. Interleukin-5 is an autocrine growth factor for Epstein-Barr virus-transformed B lymphocytes. *Blood.* 79:1763.
40. Wakasugi, N., Y. Tagaya, H. Wakasugi, A. Mitsui, M. Maeda, J. Yodoi, and T. Tursz. 1990. Adult T cell leukemia-derived factor/thioredoxin, produced by both human T-lymphotropic virus type 1- and Epstein-Barr virus-transformed lymphocytes, acts as an autocrine growth factor and synergizes with IL-1 and IL-2. *Proc. Natl. Acad. Sci. USA.* 87:8282.
41. De Waal Malefyt, R., J. Haanen, H. Spits, M.G. Roncarolo, A.T. Velde, C. Fidgor, K. Johnson, R. Kastelein, H. Yssel, and J.E. de Vries. 1991. Interleukin-10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J. Exp. Med.* 174:915.
42. Emilie, D., R. Touitou, N. Raphael, N. Peuchmaur, O. Devergnee, D. Rea, J. Coumharas, N.C. Crevon, L. Edelman, I. Joab, and P. Galanaud. 1992. In vivo production of interleukin-10 by malignant cells in AIDS lymphoma. *Eur. J. Immunol.* 22:2937.