FUNCTIONAL AND BIOCHEMICAL CHARACTERIZATION OF A SECRETED I-J(+) SUPPRESSOR FACTOR THAT BINDS TO IMMUNOGLOBULIN

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The immune system is a complex network of cell lineages and differentiated subsets that interact when exposed to foreign antigens. To simplify the study of discrete cell to cell interactions many laboratories have taken the approach of using monoclonal populations of cells as model systems in which to analyze this complex interactive network. In the B cell lineage, this has been approached through the use of B cell tumors (1, 2) and B cell hybridomas (3, 4). Similarly, for the T cell lineages, others used cloned continuous cell lines (5, 6), T cell tumors (7, 8), and T cell hybridomas (9, 10). Recently, Ricciardi-Castagnoli et al. (11) have adapted the technique of immortalizing normal functional T cells by the infection of selected normal cells with murine radiation leukemia virus, RadLV.[1] Such an approach has resulted in the production of a leukemic T cell line with a stable suppressor activity (12).

For the T suppressor cell subset a number of laboratories have described a variety of secreted factors that mediate suppressor function. These have included suppressor factors that bind antigen (13–15), factors that identify variable regions of Ig (16), suppressor factors that bind to isotypes of Ig (17), and a variety of suppressor factors that are partially encoded by genes of the MHC (13–19). The biochemical characteristics of these T cell suppressor factors are equally diverse (20–22). The precise biochemical structure of T suppressor factors (antigen-specific or nonspecific) or of the T suppressor cell recognition component remains unclear. It suggests that a variety of different factors and/or receptors are responsible for the mediation of suppressor functions in the ongoing immune response. Furthermore, the relationship between this T cell subset, its biological function, and the most recently described α, β, and γ genes that apparently encode antigen-specific T cell receptors is at this date unknown. Recent evidence

[1] *Abbreviations used in this paper:* AIDS, Acquired Immune Deficiency Syndrome; HEL, hen egg lysozyme; IgB-TsF, immunoglobulin-binding T cell suppressor factor; mIg, membrane-bound immunoglobulin; RadLV, murine radiation leukemia virus; REL, ringed-neck pheasant lysozyme.

(23) has suggested that suppressor cell lines and hybridomas do not productively rearrange, and therefore do not presumably use, these T cell receptor genes for their effector function.

In this report, we describe a suppressor factor that is secreted in nanogram quantities with high specific biological activity. The secreted factor that is a product of a T cell leukemic cell line, LH-8, we have termed Immunoglobulin-Binding T cell Suppressor Factor (IgB-TsF). The factor contains determinant(s) that can be recognized by appropriate monoclonal anti-I-J antibodies. The factor also binds to unknown determinants in the Fc portion of some Ig molecules. Furthermore, the expression of biological suppressor function is not directly correlated to the expression of the α, β, and γ chain genes of the T cell receptor.

Materials and Methods

Animals.   Age- and sex-matched BDF_1, A/J, and CAF_1 mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

Antigens.   BSA and hen egg lysozyme (HEL) were purchased from Sigma Chemical Co., St. Louis, MO. KLH was purchased from Cal Biochem-Behring, La Jolla, CA. SRBC were purchased from Scott Laboratories, Inc., Fiskeville, RI. Haptenated proteins were prepared as described (24, 25). Ring-necked pheasant lysozyme (REL) was a gift of Dr. A. Miller, University of California, Los Angeles.

Cell Lines.   The LH-8.105 line was a gift of Drs. L. Adorini (Centro Studi Nucleari Casaccia, Rome, Italy) and P. Ricciardi-Castagnoli (University of Milan, Milan, Italy) (11, 12). The two subclones, LH-8.1 and LH-8.2, were 2 of 12 subclones cloned by limiting dilution in this laboratory, and whose supernatants were screened for suppressive activity. The LH-8.1 line was shown to secrete a very active suppressor factor, while the LH-8.2 line has a minimum of 20–50 times less biological suppressor activity. Both lines have similar growth characteristics and have been shown to be free of mycoplasma. Conditioned medium from these lines was prepared by plating the cells at 5×10^5 cells/ml in RPMI 1640 medium with β-ME and 20% FCS for 48 h. The saturation density for both these cell lines is ~4 $\times$ 10^6 cells/ml, with a doubling time of 12–16 h. The supernatants were then collected and aliquots frozen or prepared immediately for purification. FCS lots were carefully screened for their ability to support growth, as well as their cell surface I-J^b determinant(s) expression.

All Ars-specific hybidomas were made and maintained in this laboratory, as previously described (26).

mAbs and Myeloma Proteins.   The monoclonal anti-I-J^b and anti-I-J^d antibodies were a gift of Dr. C. Waltenbaugh, Northwestern University, Chicago, IL. The anti-Lyt-1, anti-Lyt-2, and anti-Thy-1.2 were obtained from New England Nuclear, Boston, MA. The anti-MAC-1 monoclonal was a gift of Dr. J. Giorgi, University of California, Los Angeles. The rat anti–mouse FcR monoclonal was a gift of Dr. J. Unkeless, Rockefeller University, New York. The anti-L3T4 monoclonal was a gift of Dr. D. Raulet, MIT. The alloantisera, anti-H-2^b (B10.D2 anti-B10) was a gift of Dr. H. Eisen, MIT. The anti-HEL hybridoma proteins 523-1D10, 523-1G9, 519-2F4, AG5, 525-5E11, and 525-6D7 were a gift of Drs. E. Sercarz and A. Miller, University of California, Los Angeles. The anti-NP hybridoma proteins S43, B619, and B6P2, were a gift of Dr. T. Imanishi-Kari, MIT. These mAbs were purified by affinity chromatography (anti-Ars, anti-HEL, and anti-NP mAbs), euglobulin precipitation (anti-I-J and anti-FcR mAbs), protein A–Sepharose (anti-I-J mAbs), and/or DEAE-cellulose ion exchange chromatography (anti–FcR monoclonal).

Fab and F(ab′)$_2$ fragments of purified mAbs were prepared as previously described (27, 28). Proper digestion was confirmed by molecular sizing on Laemmli gels (29). Yields of 5–10% and 70–80% were typical for the digests of the mAbs for the F(ab′)$_2$ and Fab preparations, respectively.

Fluoresceinated Anti-Ig and Cell Staining.   All fluoresceinated anti-Ig were purchased from Cappel Laboratories, Cochranville, PA. These included fluoresceinated goat anti–

mouse Ig, F(ab')$_2$ goat anti–mouse F(ab')$_2$ Ig, and F(ab')$_2$ goat anti–rat Ig. Fluoresceinated anti-Ig reagents were adsorbed with LH-8 cells before use. All primary staining reagents were centrifuged at 100,000 g for 45 min before use to eliminate aggregated complexes. All other reagents were centrifuged at 10,000 g for 10 min before use.

10^6 cells to be tested were washed in PBS with 0.5% FCS and then incubated with the primary staining reagent (as above) for 30–45 min at 4°C. The cells were washed with PBS + 0.5% FCS and pelleted three times. A 1:10 dilution of the fluoresceinated antibodies was made (1:50 for goat anti–mouse Ig), and 100 μl of this was added to the washed pellet. Cells were resuspended, then incubated for 30 min at 4°C. Cells were again washed and resuspended for analysis in 0.5–1.0 ml of PBS with 0.5% FCS.

Quantitative flow cytometry was performed as previously described (30).

Immunoadsorbent columns. 5–10 mg of purified mAb or gamma globulin fraction of tissue culture medium was coupled with 1 g of Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) by the method of Axen et al. (31). The gamma globulin fraction from serum-free medium supernatants was used in the case of the anti-I-J mAbs.

Biosynthetic Labeling of LH-8 Suppressor Factor. A total of 50 mCi of [^{35}S]methionine (New England Nuclear) was added to 500 ml of LH-8 cells at 10^6 cells/ml in methionine-free RPMI 1640 medium with 10% dialyzed FCS. Cells were incubated for 8 h at 37°C and the supernatants harvested. Supernatants were first salted-out to 50% ammonium sulphate, and the supernatant was collected and further salted-out with 90% ammonium sulphate. The (50–90%) pellet that had been shown to retain $\geq$90% of the starting biological activity was resuspended in 1/100th of the original volume and dialyzed into PBS. This concentrate was then passed over an anti-I-J^d-coupled Sepharose column at 4°C. The effluent from this column was then passed over an anti-I-J^b-coupled column that had been equilibrated in PBS with 0.01% NP-40. The column was washed with 20 void volumes of PBS with 0.01% NP-40 and the adsorbed protein eluted with 4 M NaCl. This eluted fraction was extensively dialyzed and then concentrated. Aliquots of all fractions were counted in liquid scintillation and normalized to the original starting conditioned medium. The anti-I-J^b-eluted fraction accounted for 0.04–0.06% and 0.005–0.01% of the total biosynthetically labeled and secreted protein of the LH-8.1 and LH-8.2 subclones, respectively. The anti-I-J^b Sepharose-eluted fractions were size-fractionated under nonreduced or reduced conditions (25% 2-ME) using 13% acrylamide Laemmli gels that were then impregnated with fluor (Enlightening, New England Nuclear). Gels were first dried then incubated at −70°C for 30 d with XAR-5 x-ray film (Eastman Kodak Co., Rochester, NY) and developed.

Assays for LH-8 Suppressor Factor. The assay for in vivo suppression of the primary response has been previously described (12). Briefly, 100 μg of antigen emulsified in CFA was injected intraperitoneally on day 0. On days 1, 2, and 3 after immunization 0.5 ml of conditioned medium or purified suppressor factor was injected intravenously. Animals were killed on day 7 or 8 and the parathymic nodes dissected. Pools of lymph nodes from 3–5 animals were made and the percent of secretory cells was tested according to a modified Jerne plaque assay (32).

SRBC were coupled with antigen according to published procedures (33). Developing antisera was purchased from Gateway Immunosera, St. Louis, MO. Complement was obtained from Colorado Serum Co., Denver, CO.

The in vitro suppression assays were performed according to the microculture technique (34), and as adapted by Fridman et al. (35). Specifically for these assays, spleen cells from animals that had been primed in vivo with two injections of antigen were used 14–30 d after the last immunization. Cultures were incubated with or without antigen at 5 × 10^5 to 10^6 cells per well. 20–100-fold dilutions of sterilized fractions that were to be tested for suppressor activity were added to microtiter wells in triplicate or quadruplicate. Cultures were fed daily for the first 4 d with the nutritive cocktail of Mishell et al. (34). Plaque assays were performed on day 5 after incubation at 37°C as described above.

Preparation of Total Cellular RNA and Northern Blotting. RNA from all cell lines were prepared according to the method of Glisin et al. (36). Glyoxalation and electrophoresis of RNA was performed according to McMaster and Carmichael (37). RNA was blotted

TABLE I

Titration of Suppressor Activity of Subclones of LH-8

Strain	Immuniz-ing anti-gen*	Source of conditioned medium‡	Dilution§	Number of PFCs/10⁶ cells‖
BDF₁	HEL	None	—	3,340 ± 265
BDF₁	HEL	LH-8.1	1:10	378 ± 25
BDF₁	HEL	LH-8.1	1:100	1,899 ± 201
BDF₁	HEL	LH-8.1	1:1,000	2,833 ± 116
BDF₁	HEL	LH-8.1	1:10,000	3,244 ± 301
BDF₁	HEL	None	—	1,400 ± 119
BDF₁	HEL	LH-8.1	1:10	130 ± 8
BDF₁	HEL	LH-8.1	1:100	200 ± 37
BDF₁	HEL	LH-8.2	1:10	930 ± 117
BDF₁	HEL	LH-8.2	1:100	2,037 ± 493
A/J	HEL	None	—	4,026 ± 493
A/J	HEL	LH-8.1	1:100	4,851 ± 261

* 100 μg of antigen in a 1:1 emulsion of CFA was injected intraperitoneally on day 0.

‡ Conditioned medium was made from the subclones by plating the lines at 5 × 10⁵/ml in RPMI 1640 with 20% FCS. The cultures were incubated for 48 h at 37°C. The supernatants were harvested, immediately ali-quoted, and frozen.

§ All dilutions were performed in PBS with 1% FCS. Animals were then injected intravenously with 0.5 ml of the indicated dilution on days 1, 2, and 3 after immunization.

‖ The parathymic lymph nodes were dissected from the animals on day 7 or 8 after immunization. Pools of 3–5 animals were then assayed in triplicate for the percent of secretory cells. The numbers represent the mean ± SEM for the individual plaque assays.

to nitrocellulose and hybridized to nick-translated DNA that had incorporated ³²P-labeled nucleotides (38). Nick-translated probes were labeled to specific activities of >4 × 10⁷ cpm/μg DNA. Blots that were probed several times were recycled by incubating them in 60% formamide with 50 mM Tris (7.5) and 0.1% SDS at 60°C for 15 min. The cDNA clone (p86T5.91) for the β chain of the T cell receptor was a gift of Dr. M. Davis, Stanford University, Stanford, CA. The α (pHDS-58) and γ (HDS4/203) chain genes were provided by Dr. S. Tonegawa, MIT. The rat α tubulin probe was a gift of Dr. I. Lemischka, MIT. After the blots were washed to remove nonhybridized probe, the blots were autoradi-ogrammed overnight at −70°C with intensifying screens (Lightening; DuPont Co., Wil-mington, DE).

Results

Suppressor Activity of Subclones of LH-8. In Table I, the ability of conditioned medium from the subclones LH-8.1 and LH-8.2 to suppress the in vivo humoral response to HEL is shown. The LH-8.1 subclone showed significant suppressor activity with dilutions up to 1:100. Some suppressor activity can even be seen in the 1:1000 dilution of the conditioned medium of LH-8.1. However, as can be seen in the second section of Table I, the LH-8.2 subclone showed little if any suppressor activity at either of the dilutions presented. This was contrasted with the LH-8.1-conditioned medium, which demonstrated up to 90% suppression in similar dilutions. The lack of significant suppression by LH-8.1-conditioned

TABLE II

In Vivo and In Vitro Antigen Specificity of LH-8 Suppressor Factor

Strain	Antigen	Percent suppression at a final dilution of conditioned medium of:			
		In vivo*		In vitro‡
		1:50	1:200	1:50
BDF₁	HEL	74.6 ± 8.1 (4)§	67.3 ± 7.1 (8)	86.8 ± 8.3
BDF₁	REL	36.4 ± 4.3 (3)	12.0 ± 5.2 (3)	ND
BDF₁	SRBC	10.6 ± 3.1 (3)	9.0 ± 6.2 (2)	12.2 ± 4.8 (2)
BDF₁	TNP-BSA	59.1 ± 9.0 (3)	62.7 ± 11.5 (6)	ND
BDF₁	TNP-KLH	2.5 ± 2.5 (2)	8.0 ± 8.0 (3)	78.7 ± 5.5 (7)
BDF₁	Ars-BSA	ND	86.1 (1)	ND
BDF₁	Ars-KLH	ND	40.5 ± 20.5 (3)	75.5 ± 9.5
A/J	HEL	0	0 (2)	ND
CAF₁	TNP-KLH	ND	ND	10.1 ± 3.3 (3)

* Each experiment contained 3–5 animals per group. These figures represent the mean percent suppression as compared with the immunized control from 2–8 individual experiments. Suppressed mice were injected on day 0 with 100 μg of antigen in CFA₁ and then injected intravenously with 0.5 ml of a 1:50 or 1:200 dilution of LH-8-conditioned medium on days 1, 2, and 3.

‡ Primary antigen immunization was administered in vivo by the intraperitoneal injection of 100 μg of antigen in CFA on day 0. Animals were boosted intraperitoneally on day 30 with 100 μg of antigen in PBS. Animals were rested 14–30 d and their spleens removed. A secondary in vitro immunization with antigen was made for 5 d in the presence or absence of LH-8.1-conditioned medium (1–2% final concentration). Spleen cells were then assayed in triplicate for PFCs per well.

§ The number in parentheses represents the number of experiments in this summation. Each experiment represents the average suppression of 3–5 animals of dilutions of 1:50 or 1:200 of conditioned medium from LH-8 ± SEM. The percent suppression was calculated as [(experimental − background) ÷ (untreated control − background) × 100].

medium in humoral response to HEL in the A/J mice suggests that the suppressor factor is partially restricted in its action. This agrees with previous reports that mapped the restriction to the I region (12).

Antigen Specificity of LH-8 Suppressor Factor. A variety of different antigens tested for the ability of the LH-8 suppressor factor to inhibit a humoral response to them are shown in Table II. This ability was tested both in vivo and in vitro in BDF₁ mice. There appears to be an overall hierarchy of antigens that can be suppressed by the IgB-TsF. The suppression does not appear to be totally nonspecific, in that the humoral response to SRBC was not affected by any dilution of the factor. Other antigens, to varying degrees, were susceptible to suppression. In all cases, the HEL humoral response was the easiest to suppress. Furthermore, TNP-KLH, an antigen whose in vivo humoral response could not be suppressed, could be suppressed using the in vitro assay. In data not shown, purified preparations of IgB-TsF at concentrations 10–100 times greater than that required to show good suppression with HEL, were capable of suppressing at least part of most humoral responses to all antigens tested. It would therefore appear that IgB-TsF is "selective" but not specific in its action. This selective nature of IgB-TsF is best shown by the differences seen between the suppressions

TABLE III

Cell Surface Phenotype of LH-8 Subclones

Cell line	1° Staining reagent*	Cell surface staining[‡]
LH-8.1:	Goat anti–mouse Ig	−
	Anti-Thy-1.2 (mAb)[§]	++++
	Anti-Lyt-1 (mAb)	−
	Anti-L3T4 (mAb)	−
	Anti-Lyt-2 (mAb)	+
	Rat anti–mouse FcR (mAb)	−
	Anti-H-2^b (K^{d}αK^b)[‖]	++
	Anti-MAC-1 (mAb)	−
	Anti-I-J^b (mAb/WF9.5.2)	+/−
	Anti-I-J^b (mAb/WF9.40.5)	+
	Anti-I-J^b (mAb/WF9.1.4)	++
	F(ab')$_2$ anti-I-J^b (mAb/WF9.1.4)	++
	Anti-I-J^d (mAb)	−
LH-8.2:	Goat anti–mouse Ig	−
	Anti-Thy-1.2 (mAb)	++++
	Anti-Lyt-1 (mAb)	−
	Anti-Lyt-2 (mAb)	+/−
	Anti-I-J^b (mAb/WF9.40.5)	+/−
	Anti-H-2^b (K^{d}αK^b)	++

* Purified anti-I-J mAbs were diluted to ~1 mg/ml for primary staining of cells. 20 μl of reagents was added to 100 μl of PBS with 0.5% FCS containing 10^6 LH-8 cells. Other mAbs were added at concentrations that were twice the amount known to be optimal for positive cell populations. The second-step reagent in all cases was an F(ab')$_2$, fluoresceinated reagent of the appropriate specificity, which had been adsorbed with LH-8.1 cells. All analyses were quantitated by flow cytometry on either a Cytofluorograph H-50 (Ortho Diagnostic Systems Inc., Raritan, NJ) or a B-D/FACS-III (Becton Dickinson & Co., Mountain View, CA).

‡ Negative controls for these reagents were MOPC-21-purified protein (10 μl of 1.0 mg/ml) and the second-step fluoresceinated reagents. −, a shift in peak fluorescence channel of less than three channels and less than 3% cells positive when compared with the negative control. +/−, a shift of 3–5 channels of peak fluorescence and/or 3–5% of cells considered positive. +, a peak fluorescence channel shift of 5–7 channels and/or 10–20% of the cells clearly positive. ++, a shift of 7–10 channels and/or 20–40% of the cells were positive. ++++, all cells were very strongly stained.

§ mAb indicates that the primary staining reagent was an mAb of the indicated specificity.

‖ Alloantisera from B10.D2 mice were raised by the repetitive immunization of these mice with B10 spleenocytes.

when HEL or REL are used as antigens. These two lysozymes are very closely related in amino acid structure and yet display totally different susceptibilities to the suppression of their humoral response. This data shows the necesity of titrating "suppressor" factors that may appear to be specific in order to clearly define the level of specificity or restriction.

Cell Surface Phenotype of LH-8.1 and LH-8.2. In Table III a summary of the cell surface phenotype of the subclones LH-8.1 and LH-8.2 is shown. The cell surface phenotype of these subclones clearly showed that they are T cells and
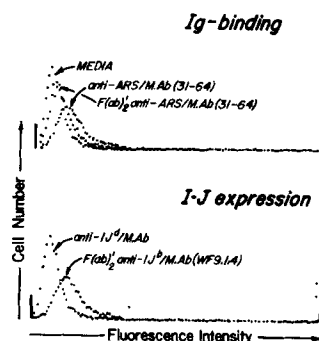
Ig-binding

I-J expression

Figure 1. Surface-staining of LH-8.1 cells with anti-I-J and other mAbs. 10^6 LH-8. cells in 100 μl PBS were incubated for 45 min at 4°C with 30 μl of anti-I-J^d, F(ab')$_2$/WF9.1.4 (anti-I-J^b), 31-64 (anti-Ars), or F(Ab')$_2$ 31-64 at concentrations of 1 mg/ml. The cells were washed three times with PBS containing 0.5% FCS, and then 20 μl of fluorescenated F(ab')$_2$ goat anti–mouse IgG-F(ab')$_2$ was added and allowed to incubate 30 min at 4°C. Cells were washed and analyzed on a B-D FACS III. Gains were set so the peak of the fluorescence profile for the autofluorescence controls was contained within the first 10 channels.

that the LH-8.1 line expresses the I-J^b determinant, albeit weakly, on its cell surface. This is shown by the positive reaction with three separate anti-I-J^b mAbs. The possibility that the staining with the anti-I-J^b mAbs was due to nonspecific adherence to the cell surface via the constant region of the antibody was ruled out because of the positive results with the F(ab')$_2$ fragment of the WF9.1.4 monoclonal (Fig. 1). It was necessary to use the divalent F(ab')$_2$ because of the apparent weak interaction of the monoclonal with the cell surface I-J determinants. The lack of staining with the anti-I-J^d mAb confirmed the specificity of the cell surface staining. The LH-8.2 subclone exhibited a very similar cell surface phenotype; however, in parallel experiments there appeared to be a diminution of the cell surface determinants for I-J. This result correlated with this subclone's inability to secrete a functional suppressor factor compared with the LH-8.1 subclone. In data not shown, the expression of the I-J^b determinant on the LH-8.1 line was dependent on the growth cycle of the subline and the lot of FCS. FCS lots had to be carefully screened for their ability to support the cell surface expression of I-J^b, independent of the ability of individual FCS lots to support the growth of the sublines.

Binding of Various Hybridoma Proteins to the Cell Surface and Secreted Suppressor Factor Product of LH-8.1. In early experiments it was observed that some hybridoma proteins, originally used as controls for immunofluorescence studies, seemed to bind to the surface of LH-8.1. In Table IV a collection of various hybridoma proteins that contain different antigen specificities and different isotypes were examined for their ability to bind to the cell surface of the LH-8.1 line (see Fig. 1). They were also examined for their ability to bind the secreted suppressor factor. A direct correlation was shown to exist between the phenomena of binding Ig to the cell surface of LH-8.1, and whether or not Ig coupled to Sepharose can adsorb the factor out of conditioned medium. Furthermore, no obvious correlation can be made with the factor's ability to bind to distinct heavy or light chain isotypes, allotypes, or idiotypes, or to known structural genes responsible for antibody formation. In the latter part of Table IV, it is shown

TABLE IV

Binding of Hybridoma Proteins to the Cell Surface and Secreted Suppressor Factor of LH-8.1

Hybridoma	Specificity	Isotype	Reactivity	
			Cell surface*	Secreted factor[‡]
36–65	Ars	γ_1, κ	++	+
45–165	Ars	γ_{2b}, κ	+	+
45–49	Ars	γ_{2b}, κ	++	+
31–64	Ars	γ_{2b}, κ	+	ND
36–54	Ars	γ_{2b}, κ	–	–
36–60	Ars	γ_{2a}, κ	–	–
31–62	Ars	γ_{2a}, κ	–	–
31–41	Ars	γ_1, κ	–	–
523–1D10	HEL	γ_1, κ	+	+
523–1G9	HEL	γ_1, κ	–	–
519–2F4	HEL	γ_1, κ	–	ND
AG5	HEL	γ_1, κ	–	ND
525–5E11	HEL	γ_1, κ	–	ND
552–6D7	HEL	γ_1, κ	–	ND
S43	NP	γ_{2a}, λ_1	+	ND
B619	NP	γ_{2a}, λ_1	–	ND
B6P2	NP	μ, λ_1	–	ND
36–65 Fab	Ars	γ_1, κ	–	–
31–64 (Fab')₂	Ars	γ_{2b}, κ	–	ND

* 10^6 LH-8.1 cells were first incubated with 20 μl of hybridoma protein (1 mg/ml) in a total volume of 100 μl PBS with 0.5% FCS. Cells were washed and then incubated with 5–10 μl of the F(ab')₂ fragment of fluorescenated goat anti–mouse Ig. This second-step reagent was first adsorbed with 10^7 LH-8.1 cells per 100 μl. Stained cells were analyzed by quantitative flow cytometry. –, a shift in mean fluorescence of <3 channels and <3% cells positive as compared with a negative control using either MOPC-21-purified paraprotein or 31–62 hybridoma protein. +, a peak fluorescence channel shift of 5–7 channels and 10–20% of the cells considered to be clearly positive. ++, a 7–10 peak channel shift and 20–40% of the cells clearly positive.

‡ +, 90% of the suppressor activity from conditioned medium could be adsorbed and subsequently eluted from the affinity column.

that the binding to the cells and the factor can be abolished if the Fab fragment of the mAb is used. In addition, an mAb, 31-64, which had exhibited cell surface binding reactivity (see Table IV and Fig. 1) with LH-8.1, also lost this ability when one made the F(ab')₂ of this mAb (Fig. 1). This data shows that the suppressor factor has an affinity for a determinant in the Fc region of the heavy chain. Complex carbohydrates would seem to be likely candidates to account for such differences. Alternatively, the Fc portion may be necessary to stabilize structural epitopes in the variable region of the Ig molecule.

Affinity Adsorption of LH-8.1 Suppressor Factor. Since there appeared to be a restricted specificity for the suppressor factor and the cell surface of LH-8.1-expressed I-J determinants, we wanted to find out whether the IgB-TsF contained determinants that could be recognized by anti-I-J mAbs. Purified anti-I-J mAbs were coupled to Sepharose 4B and conditioned medium was passed over these

TABLE V

Affinity Purification of LH-8.1 Suppressor Factor

Hybridoma-coupled Sepharose 4B	Relative suppression*	
	Effluent[‡]	Eluate[§]
Anti-I-J[d]	++++	−
Anti-I-J[b] (WF9.40.5)	+/−	++++
Anti-Ars (45–49)	−	++++
Anti-Ars (36–65)	+	+++
Anti-I-J[b] (WF9.40.5) + anti-Ars (36–65)	+/−	++++

* Effluent and eluates from all columns were tested using the in vivo assay with three log dilutions. −, no suppression was seen. +/−, a small amount of suppression was seen in the undiluted fractions. +, some suppression was seen in the undiluted fraction and the first dilution. +++, ~70–90% of the reactivity was recovered in this fraction and significant suppression could be seen in even high dilutions. ++++, as much suppression was seen in this fraction in all dilutions as compared with a control of conditioned medium. Presumably, this accounts for ≥90% of the biological activity.

‡ Columns were equilibrated with PBS containing 0.05% FCS. A sample of concentrated LH-8.1-conditioned media (20 X) was loaded on each column. After discarding one void volume, two void volumes were collected. The columns were then washed with 10–20 elution volumes of PBS before salt elution. Samples were dialyzed against PBS before assay.

§ 4 M NaCl was used to elute any biological activity. A volume equal to two times the loading volume was passed over the column and the salt-eluted fraction was collected and dialyzed to an equivalent starting conditioned medium concentration for assay.

respective columns. Line 1 of Table V shows that the anti-I-J[d]-coupled Sepharose column exhibited all the suppressor activity in the effluent. However, the monoclonal anti-I-J[b] Sepharose column bound the suppressor factor and this activity could be eluted from that column, as seen in line 2. Similarly, it was shown that the factor could also be bound to, and eluted from, the column coupled with the anti-Ars hybridomas, 36-65 and 45-49. It was of interest to ask whether there was a single or several distinct factor(s) that bound to the anti-I-J column and the anti-Ars hybridoma–coupled columns.

In line 5 of Table V, it is shown that the anti-I-J[b]-eluted factor was also found to bind to the anti-Ars (36-65) column. This suggested that a single suppressive molecule (perhaps multiple chains) was responsible for the binding to Fc portion of Ig and contained an I-J determinant.

Biosynthetic Labeling and Purification of LH-8 Suppressor Factor. To show that the LH-8 cells were indeed synthesizing and secreting the suppressor factor, cells were biosynthetically labeled with [³⁵S]methionine. Since we had shown that the two LH-8 subclones have distinct biological activities in terms of quantitative suppression, we felt we were able to follow the specific proteins associated with suppression by comparing the labeled material from these two subclones. Biosynthetically labeled material from both subclones was concentrated and passed over an anti-I-J[d]-coupled Sepharose column. The effluent was then passed over an anti-I-J[b]-coupled Sepharose column and the adsorbed material eluted and con-
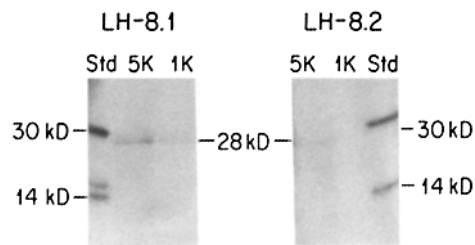
FIGURE 2.   Purification of biosynthetically-labeled LH-8 suppressor factor. Both proteins of LH-8.1 and LH-8.2 were biosynthetically labeled with [^{35}S]methionine for 8 h at 37°C. Superantants were first salted-out to 50% ammonium sulphate and the pellet discarded. The supernatants were then salted-out to 90% ammonium sulphate and the pellet collected and diluted in PBS. These concentrates were then dialyzed into PBS and passed over an anti-I-J^d/ mAb-coupled Sepharose 4B column and washed with 20 void volumes of PBS with 0.01% NP-40. This effluent was then passed over with 20 void volumes of PBS with 0.01% NP-40. The eluted factors were eluted with 4 M NaCl. Eluted fractions were dialyzed into PBS and concentrated. The eluted fractions represented 0.004 and 0.001% of the total biosynthetically labeled protein of the LH-8.1 and LH-8.2, respectively. Purified fractions were then loaded on 13% Laemmli gels under nonreduced and reduced conditions and impregnated with fluor. Gels were autoradiographed with x-ray film at −70°C for 30 days and the films were developed.

centrated. Concentrated material from both sublines were electrophoresed through 13% acrylamide gels, then subjected to fluorography.

The factor from the LH-8.1 subclone showed a complex pattern of bands with multiple molecular masses. However, upon reduction, a single band was noted of approximately 28 kD (Fig. 2). Furthermore, this band was also seen in the LH-8.2-purified fraction but represented ~5–10-fold less material. It was also observed that three- to fivefold less total biosynthetically labeled protein was adsorbed to the anti-I-J^b column from the LH-8.2 subclone supernatant vs. the biosynthetically-labeled material from the LH-8.1 subclone. Therefore, collectively these data suggest that the suppressor factor from the LH-8.1 subclone was indeed a single chain of ~28 kD and that this protein was made in an ~20–50-fold less amount in the LH-8.2 subclone. This agreed with the biological assays for these two subclones.

Expression of the α-, β-, and γ-associated Genes of the Normal T Cell Receptor in the LH-8.1 and LH-8.2 Subclones.   Since LH-8 exhibited a phenotype that correlated with a suppressor T cell subset, we were interested in whether the two subclones of LH-8 expressed the T cell receptor genes. In Fig. 3 A–C it can be seen that both the LH-8.1 and LH-8.2 express the $C_{T\alpha}$, $C_{T\beta}$, and $C_{T\gamma}$ gene messenger RNA, and, to the first approximation, in equivalent quantities. The control for proper quantitative loading of intact mRNA is seen in Fig. 3 D. This blot was probed with the rat α tubulin probe as a positive control for all cell lines. Since the LH-8.1 and LH-8.2 differ ~20–50-fold in expression of suppressor function, as well as the expression of the 28 kD protein, it might have been expected that the expression of the T cell receptor genes would correlate with the expression of the presence or absence of suppressor function. This is clearly not the case for this T cell. However, the LH-8.1 cell line is an example of a T cell that retains a suppressor cell phenotype, and at the same time rearranges and expresses the $C_{T\alpha}$, $C_{T\beta}$, and $C_{T\gamma}$ genes. Previously, only T cells of the helper (39) or cytotoxic (40) cell phenotype have been described to productively rear-
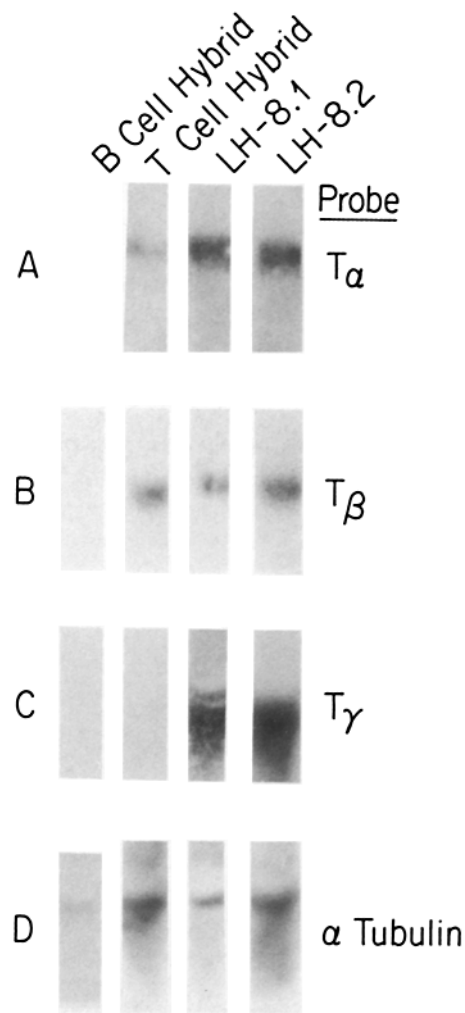
FIGURE 3.   Northern blot analysis of expressed genes of LH-8 subclones. Expression of T cell receptor genes in LH-8.1 and LH-8.2. RNA was isolated from a B cell line (A20), a macrophage line (P388.D1), and a Th hybridoma (6.2), as well as the LH-8.1 and LH-8.2 subclones. 40 μg of total RNA from each line were glyoxalated and electrophoresed overnight through 1.2% agarose gel. The gels were blotted with nitrocellulose and the blots were hybridized with nick-translated, ^{32}P-labeled $C_{T\alpha}$, $C_{T\beta}$, $C_{T\gamma}$ or α-tubulin probes. The blots were washed and then autoradiogrammed overnight with enhancing screens at −70°C. Molecular weight markers are in the margins.

range and transcribe these T cell receptor genes. A recent report also has suggested (41) that the parental LH-8.105 cell line expresses the appropriate antigen-specific T cell receptor dimer on its surface. It is interesting to note that both the LH-8.1 and LH-8.2 line express high levels of full length $C_{T\gamma}$ mRNA. This gene has only been described to be expressed on cytotoxic T cells (42). However, the only phenotypic marker that distinguishes murine suppressor T cells from cytotoxic cells is the I-J determinant, and the precise molecular basis for this determinant has most recently been questioned (43).

Discussion

Since the original description of suppressor cells (44), a number of laboratories have tried to biochemically characterize the factor responsible for immuno-suppression. These efforts have yielded a large family of factors that are capable of mediating suppression. These factors have equally diverse characteristics, in that some bind antigen (20–22); some bind Ig idiotypic determinants (45); some express idiotypic determinants (16); some bind the Fc portion of Ig (17); and some contain by themselves, or in combination with other chains, an MHC-encoded determinant. Perhaps the lack of precise biochemical data on the nature of many suppressor factors, even with the availability of cloned hybridomas and cell lines, is in part due to their inherent high specific activity and relative lability, very analogous to some peptide hormones.

In this study, we describe a factor, which in nanogram quantities of protein is capable of suppressing the in vivo or in vitro humoral response to a variety of different antigens. In terms of its biological function, it seems to most closely resemble the immunoglobulin-binding factor (IBF) of Fridman et al. (35). Our factor, IgB-TsF, does seem to bind to several randomly selected hybridoma proteins, and like IBF this binding is restricted to the Fc portion of these mAbs. This was shown in this study by the lack of adsorption of the factor to an Fab fragment–coupled Sepharose column, whereas the factor had been shown to bind to the column coupled with the intact hybridoma protein. However, unlike IBF, IgB-TsF is *not* isotype-specific in its binding to various Ig. We have preliminary evidence to suggest that the binding to the Fc region is due to glycosylation of this portion of the Ig molecule. This suggests that the recognition portion of this suppressor molecule might be "lectin-like". Differential glycosyl moieties on the surface of various subsets or activation stages of lymphoid or presenting cells might serve to localize and concentrate the action of such a suppressor factor. In studies not shown, we have also shown that, unlike IBF, our factor cannot be "up-regulated" (46) by incubation with hybridoma proteins that bind the secreted factor. Also, IgB-TsF cannot directly inhibit the secretion of Ig of hybridomas that bind the factor. The lack of H-2 restriction in the mediation of the suppression for the IBF (17) is also, in part, at odds with the characteristics of IgB-TsF, although such an observation could merely be dose-related. However, a common feature of IgB-TsF and IBF is that they are both associated with I region determinants. The molecular mass characteristics of soluble IBF and IgB-TsF are also distinct, i.e., 40 kD vs. 28 kD.

In terms of its biochemical characteristics, IgB-TsF seems to resemble the light chain of Tada et al. (20) or the single chain factor of Krupen et al. (21), with several distinct differences. Unlike the factor of Tada et al., the IgB-TsF is only composed of a single peptide chain. However, like the light chain component of the factor of Tada et al., it is a 28 kD chain that contains determinants recognized by anti-I-J mAbs. This light chain is also responsible for the mediation of the suppressor function, although it has no effect unless associated with the 45 kD, antigen-binding chain.

The factor of Wieder et al. (47) has been described as a single peptide chain that can bind antigen and contains an I-J determinant; this factor has a minimal molecular mass of ~24 kD. The IgB-TsF in this paper would seem to closely

resemble this factor, except for its lack of antigen-binding characteristics. However, the original clone, LH-8.105, from which the two subclones, LH-8.1 and LH-8.2, were derived, had been reported to secrete an antigen-specific suppressor factor (12). Since the two subclones were originally selected on the basis of the presence or absence of suppressor activity to the in vivo humoral response to HEL and not for their antigen specificity, perhaps the LH-8.1 subclone represents a mutated (in terms of antigen specificity), antigen-binding moiety of a factor analogous to Wieder et al. (47). Our limited biochemical studies have suggested that our factor is not lipoprotein, but is possibly a glycoprotein.

The factor could also be a "light chain" only secretor of the factor of Tada et al. (20). This is clearly a common event in B cell lines and hybrids. In this case, however, the factor also would have gained the ability to express biological suppressor activity as a single chain.

Regardless of the relationship of this factor to others previously reported, it shows several points of significance in the elucidation of the biochemistry of secreted suppressor factors. First, the ability to bind or not bind mAbs is not, in and of itself, sufficient evidence for the presence of the mAb-defined determinant on the suppressor molecule. The use of F(ab')$_2$ fragments of these mAbs can clearly distinguish betweeen the Ig-binding property, as exhibited by our IgB-TsF, and the binding due to variable region antigen recognition. Similarly, one must also extensively examine the nature of isotypic (17), idiotypic (16), or anti-idiotypic (45) determinants associated with T cell suppressor factors. Alternatively, one could argue that such relationships do in fact exist for our IgB-TsF, but that such epitopes are only recognized by the intact tertiary structure of the Ig that is destroyed when F(ab')$_2$ or Fab fragments are made. The further purification and structural determination of the IgB-TsF will hopefully resolve this question.

Secondly, the antigen "specificity" of suppressor factors must clearly be defined over a wide concentration, as well as with multiple and diverse antigens. In the case of the IgB-TsF, substantial data could be presented to support "antigen-specificity," "isotype or idiotype-specificity," etc. Furthermore, although the overall effect of suppression in the host may indeed be specific, this does not mean that the mediators of suppression must also be specific. A specific recognitive "triggering" event that elicits a nonspecific, or perhaps as in the case of IgB-TsF, selective suppressor factor would also be scored as specific at the level of the whole animal.

In part, the molecular basis for I-J subregion determinants on the cell surface and associated suppressor factors has been called into question (43), although some explanation has been offered (48). A question that can be addressed using a cloned T cell line that expresses I-J determinants is: What is the relationship between the secreted I-J(+) suppressor factors, i.e. between the biological function and the previously described T cell receptor genes (39, 40)? Perhaps suppressor cells are merely differentiated stages of helper or cytotoxic cells (excess help or self killing). A great deal of debate exists over whether T suppressor cells even use the same family of genes as other T cell subsets for recognitive events associated with the elicitation of effector function. Only Th and cytotoxic T cells have been clearly inferred to use this family of genes. The

$C_{T\alpha}$, $C_{T\beta}$, and $C_{T\gamma}$ genes are rearranged and expressed for both subclones of LH-8 reported in this paper. It would also appear that either IgB-TsF secretion is totally unrelated to the expression of the T cell receptor, of if they are indeed directly linked, then the loss of suppressor function in LH-8.2 is due to posttranscriptional control of IgB-TsF expression. The cloning of the gene for IgB-TsF expression should clearly distinguish these possibilities. It is possible that although suppressor cells may indeed use a similar family of T cell receptor genes for their recognitive events associated with their activation, they might not require an intact receptor to maintain their effector function of suppression. It may be productive to concomitantly use the LH-8.1 and LH-8.2 subclones as + and − populations to further study the gene(s) associated with suppression.

The origin of the LH-8 murine T cell leukemia, Rad-LV infection, and its associated suppressor function are also of topical interest. The recent reports (49) that HTLV-III is the etiological agent of Acquired Immune Deficiency Syndrome (AIDS) may be related to the studies in this report. Both the Rad-LV and HTLV, biologically similar retroviruses, may share a tropism for certain T cell subsets. Although the target cell in this disease appears to be the Th subset, immortalizing and expanding a T cell clone or related regulatory clones with properties very analogous to the LH-8 murine leukemia could conceivably enhance or further provoke an immune deficiency state very similar to that of AIDS patients. Perhaps this murine system may be useful as a model to study such immunodeficient states. However, this also raises another point. Is the mediator of the suppression a product of a normal or aberrantly expressed T cell product; is it a product of the retrovirus; or perhaps even a chimeric product of the retrovirus, and a normal T cell product? It is already known that retrovirally infected cells often secrete an active, immunologically suppressive protein, P-15, which is a product of the retrovirus (50, 51). Furthermore, a cis or trans-activation of suppressor factor gene(s) might occur (52). Although its biochemical and biological characteristics appear to be unrelated to IgB-TsF, a chimeric gene of normal T cells and a retroviral protein might be suitable candidates for IgB-TsF, as well as previously described suppressor factors. The cloning of the gene(s) responsible for suppression in the LH-8 T cell leukemia will answer these questions.

Summary

A secreted product of a T cell leukemic cell line, LH-8, was examined for its biochemical and biological properties. The factor that we have termed Immunoglobulin-Binding T cell Suppressor Factor (IgB-TsF) was shown to be suppressive for the in vitro and in vivo humoral response to a variety (but not all) antigens tested. The cell surface phenotype of the LH-8.1 subclone was M.Ig(−), Thy-1(+), L3T4(−), Lyt-2(+), FcR(−), MAC-1(−), and H-2^b(+). In addition, both the cell surface and secreted factor, IgB-TsF, of LH-8.1 expressed determinants that were recognized by anti-I-J^b mAbs but not by an anti-I-J^d monoclonal. The same factor also retained an affinity for the Fc portion of ~30% of randomly selected, purified mAbs. This binding could be abolished if the Fab or F(ab')$_2$ fragments of these mAb were used, but was found to be unrelated to isotype of the respective mAbs. Using subclones that expressed quantitative differences in

their ability to exert suppression as sources of biosynthetically labeled IgB-TsF, we have shown the suppressor activity correlated with a single, 28 kD protein. Furthermore, comparisons of these same subclones that differ in their suppressor activity, do not show any direct correlation of this biological activity with the expression of the previously described T cell receptor genes. It also suggests that at least some suppressor cell subsets may use the same or related family of T cell receptor genes for their recognitive stage of activation as helper and cytotoxic T cell subsets, but not for their effector stage of immunologic suppression.

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