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Immunoregulatory properties of ISG15, an interferon-induced cytokine

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ABSTRACT **ISG15 is a 15-kDa protein of unique primary amino acid sequence, which is transcriptionally regulated by interferon (IFN) α and IFN-β. Because it is synthesized in many cell types and secreted from human monocytes and lymphocytes, we postulated that ISG15 might act to modulate immune cell function. ISG15 stimulated B-depleted lymphocyte proliferation in a dose-dependent manner with significant proliferation induced by amounts of ISG15 as low as 1 ng/ml (58 pM). Maximal stimulation of [^{3}H]thymidine incorporation by B-depleted lymphocytes occurred at 6–7 days. Immunophenotyping of ISG15-treated B-depleted lymphocyte cultures indicated a 26-fold expansion of natural killer (NK) cells (CD56$^+$). In cytotoxicity assays, ISG15 was a potent inducer of cytolytic activity directed against both K562 (100 lytic units per 10^6 cells) and Daudi (80 lytic units per 10^6 cells) tumor cell targets, indicating that ISG15 enhanced lymphokine-activated killer-like activity. ISG15-induced NK cell proliferation required coculturing of T and NK cells, suggesting that soluble factor(s) were required. Measurement of ISG15-treated cell culture supernatants for cytokines indicated production of IFN-γ (>700 units/ml). No interleukin 2 or interleukin 12 was detected. IFN-γ itself failed to stimulate lymphocyte proliferation and lymphokine-activated killer cell activation. Further, induced expression of IFN-γ mRNA was detected by reverse transcription–PCR in T lymphocytes after ISG15 treatment but not in NK cells. Enhancement of NK cell proliferation, augmentation of non-major histocompatibility complex-restricted cytotoxicity, and induction of IFN-γ from T cells identify ISG15 as a member of the cytokine cascade and suggest that it may be responsible for amplifying and directing some of the immunomodulatory effects of IFN-α or IFN-β.**

Although molecular mechanisms underlying biological responses to interferons (IFNs) are only partially dissected, it is thought that they are mediated by the regulated synthesis of induced proteins (1, 2). One of these IFN-induced gene products is ISG15 (3, 4). ISG15 is a 15-kDa protein that is transcriptionally regulated by IFN-α or IFN-β (5, 6). ISG15 is synthesized in mammalian cells as a 17-kDa precursor (pre-ISG15) that is processed by a cellular converting enzyme, cleaving the 8 carboxyl-terminal aa to yield the 157-aa mature ISG15 (4, 7), which is secreted from monocytes and lymphocytes (8). Both native and recombinant ISG15 induce the synthesis and secretion of IFN-γ from B-depleted lymphocytes (9).

Based on its cytokine-like properties, it was hypothesized that ISG15 modulates immune effector cell activation and function. To evaluate this, effects on peripheral blood lymphocytes were assessed by [^{3}H]thymidine incorporation, immunophenotyping, and cytolytic assays. We demonstrate that ISG15 induced production of IFN-γ from T cells, augmented the proliferation of natural killer (NK) cells, and induced non-major histocompatibility complex-restricted cytolysis of tumor cell targets by NK-derived lymphokine-activated killer (LAK) cells in the absence of detectable levels of interleukin (IL) 2 or IL-12.

MATERIALS AND METHODS

Materials. B-depleted peripheral blood lymphocytes (PBLs) were purified by passage over a nylon wool column (9). By flow cytometry, this population was 80–85% CD3$^+$, 5–8% CD16$^+$, <2% CD14$^+$, and <2% CD19$^+$. Human recombinant IL-2 (2×10^7 units/mg) was from Hoffmann-LaRoche. Human recombinant IFN-γ (2×10^7 units/mg) was from Biogen. A polyclonal antiserum to homogenous ISG15, purified from cytoplasms of IFN-β-treated Daudi cells, was raised in a New Zealand White rabbit (7). Antigenic specificities of monoclonal antibodies used for flow cytometry included CD3 (Leu 4), CD14 (Leu M3), CD16 (Leu 11a), CD19 (Leu 12), and CD56 (NKH-1) (Becton Dickinson).

Expression and Purification of ISG15 and pre-ISG15. Human 157-aa ISG15 and the 165-aa pre-ISG15 were expressed and purified from *Escherichia coli* BL21(DE3) (10, 11). Endotoxin levels were <0.03 endotoxin units (EU)/μg as measured by limulus amoebocyte lysate assay (sensitivity, 0.01 EU/ml). Heat-denatured ISG15 was ISG15 boiled for 20 min.

Functional Assays. Proliferation ([^{3}H]thymidine incorporation) assays were performed essentially as described (12). Results were reported as mean cpm ± SEM of triplicate determinations. NK/LAK cell activity was evaluated using ^{51}Cr release cell-mediated lympholysis assays (12).

Immunophenotyping. Analysis of cell surface antigens was performed by two-color immunofluorescent flow cytometry using standard direct staining methods (12). Results were calculated as the percentage of positive cells after subtraction of values for nonspecific isotype controls (fluorescein isothiocyanate-IgG2a and phycoerythrin-IgG2a) and converted to absolute cell number.

Detection of Cytokines by Immunoassay. IFN-γ was detected by radioimmunoassay (Centocor) with a sensitivity of 0.1 units/ml. IL-2 and IL-12 were assessed by ELISA (R + D Systems) with sensitivities of 6 pg/ml (IL-2) and 5 pg/ml (IL-12).

Immunomagnetic Separation of CD3$^+$ T Cells and CD56$^+$ NK Cells. PBLs were separated by the use of a commercially available NK cell isolation kit, which isolated NK cells by negative enrichment using a magnetic cell sorting separator (Miltenyi Biotec, Sunnyvale, CA). Typical yields and purity (assessed by flow cytometry) were as follows: 2×10^6 cells in NK fraction (65–75% CD56$^+$CD3$^-$, <1% CD3$^+$CD56$^-$), 5 ×

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Abbreviations: IFN, interferon; NK, natural killer; LAK, lymphokine-activated killer; PBL, B-depleted peripheral blood lymphocyte; IL, interleukin; EU, endotoxin units; RT-PCR, reverse transcription–PCR; CML, cell-mediated lympholysis; LU_{30}, lytic units 30%.

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10^5 cells in wash fraction (40–50% $CD3^+$, 40–50% $CD56^+$), and 5×10^7 cells in the T-cell fraction (90–95% $CD3^+CD56^-$, <2% $CD56^+CD3^-$).

Isolation of RNA and Reverse Transcription–PCR (RT-PCR). Total cellular RNA was extracted using RNAzol B (Cinna Scientific, Friendswood, TX) (13). Fifty nanograms of total RNA was reverse transcribed, and PCR was performed using IFN-γ-specific or β-actin-specific primers (0.2 μM) (Clontech) (14).

RESULTS

ISG15 Induced PBL Proliferation. To determine whether ISG15 acted as a mitogen to lymphocytes, human ISG15 was added to PBL cultures for 72 hr before radiolabeling with [^{3}H]thymidine for 12 hr. ISG15 increased [^{3}H]thymidine incorporation in these cultures in a dose–responsive manner; significant proliferation ($P < 0.02$ compared to controls) was observed at 1 ng/ml (58 pM) (Fig. 1*A*). The ED_{50} was 865 ± 193 pM. Specificity of biological activity of ISG15 was assessed after heat denaturation and treatment with a neutralizing antiserum raised against native ISG15 (7). Heat denaturation (Fig. 1*A*) or addition of ISG15-specific IgG (Fig. 1*B*) reduced proliferation to the baseline level.

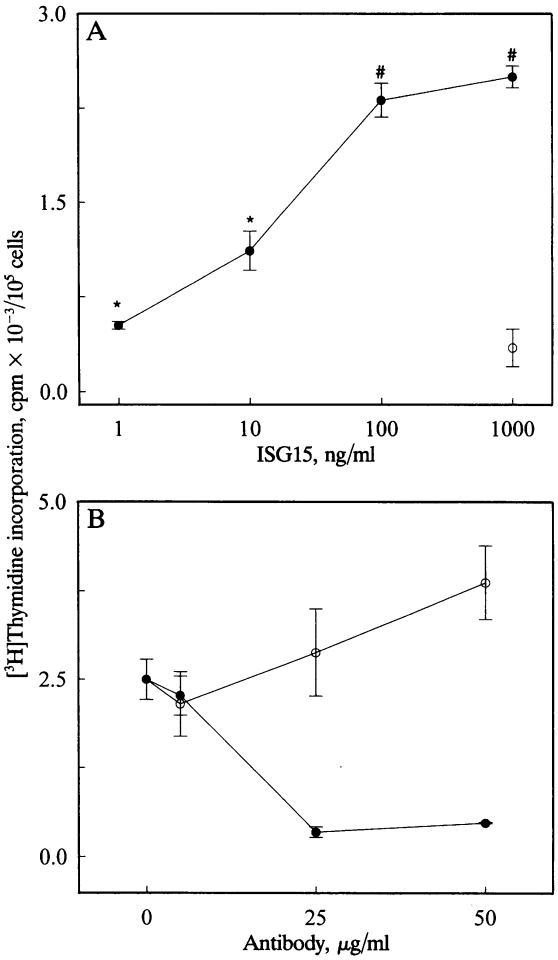

FIG. 1. Regulation of PBL proliferation by ISG15. (*A*) PBLs were cultured with various doses of purified human ISG15 (●) or heat-denatured ISG15 (1000 ng/ml) (○), and [^{3}H]thymidine incorporation was measured after 72 hr (*, $P < 0.02$; #, $P < 0.01$). (*B*) Specificity of the proliferative response of PBLs in the presence of ISG15 (100 ng/ml) with various doses of an IgG fraction of a polyclonal rabbit antibody to native ISG15 (●) or normal rabbit serum (○). Proliferation was assessed by [^{3}H]thymidine incorporation as in *A*. Baseline proliferation was 34 ± 1.9 cpm.

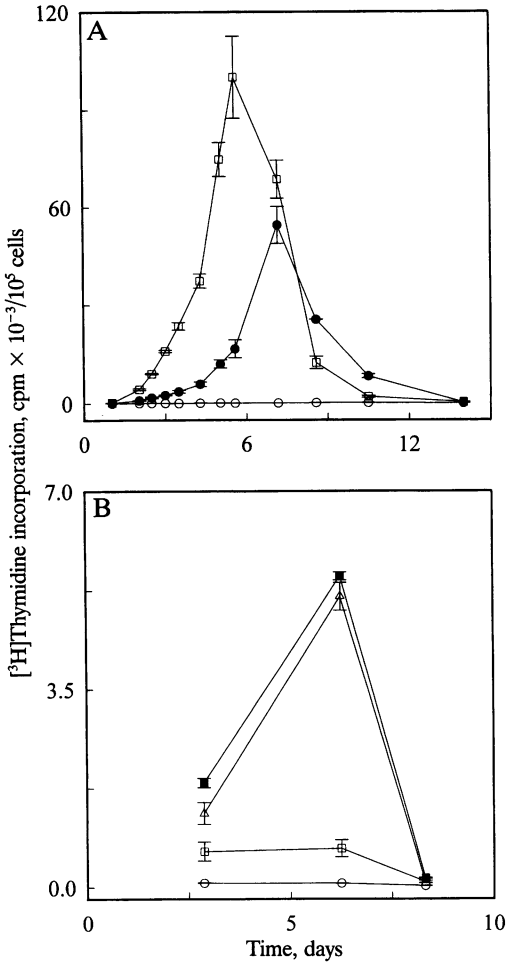

FIG. 2. Kinetics of the proliferative response of PBLs to ISG15 and IL-2. (*A*) PBLs were cultured with ISG15 (100 ng/ml) (●) or human IL-2 (100 units/ml) (□) or were untreated (○). Proliferation was assessed by a 12-hr [^{3}H]thymidine incorporation. (*B*) The proliferative response of PBLs to ISG15 (100 ng/ml; ■), ISG15 (100 ng/ml) plus an IgG fraction of a polyclonal rabbit antibody to native ISG15 (50 μg/ml; □), ISG15 (100 ng/ml) plus normal rabbit serum (50 μg/ml; △), or no treatment (○). Proliferation was assessed by a 12-hr [^{3}H]thymidine incorporation.

To assess growth kinetics of PBLs treated with ISG15, PBLs were incubated with or without ISG15 for various periods of time and radiolabeled for 12 hr with [^{3}H]thymidine. Maximal stimulation of [^{3}H]thymidine incorporation by PBLs occurred at 6–7 days (Fig. 2*A*). Neutralizing antiserum to ISG15 com-

Table 1. Influence of human pre-ISG15, ISG15, ubiquitin, IFN-γ, and murine ISG15 on PBL proliferation

Treatment	[^{3}H]Thymidine incorporation, cpm ± SEM per 10^5 cells	
	4 days	6 days
Untreated	88 ± 15	91 ± 12
Human ISG15	1844 ± 82	12,654 ± 1,289
Human pre-ISG15	70 ± 14	81 ± 9
Murine ISG15	92 ± 19	94 ± 15
Human ubiquitin	92 ± 4	75 ± 5
Human IFN-γ	93 ± 6	105 ± 5

PBLs (10^6 cells per ml) were cultured with human ISG15 (10^3 ng/ml, 58 nM), human pre-ISG15 (10^3 ng/ml, 56 nM), murine ISG15 (10^3 ng/ml, 58 nM), human ubiquitin (500 ng/ml, 58 nM), or human IFN-γ (10^3 units/ml). Proliferation was assessed by a 12-hr [^{3}H]thymidine incorporation.

Table 2. Immunophenotyping of PBL cultures treated with ISG15

Treatment	Time, day(s)	Cells in 1 ml of culture × 10^{-6} Total	$CD3^+$	$CD4^+$	$CD8^+$	$CD56^+$
Pretreatment	0	0.97	0.76			0.06
− ISG15	8	0.71	0.66	0.51	0.17	0.01
+ ISG15	8	1.2	0.8	0.54	0.23	0.26

PBLs (10^6 cells per ml) were treated with or without ISG15 at 100 ng/ml. At the indicated time, cells were removed from culture and counted, and two-color direct immunofluorescence was performed.

pletely blocked ISG15-induced PBL proliferation; normal rabbit serum had no inhibitory effect (Fig. 2*B*). ISG15-induced PBL proliferation was compared to IL-2-induced proliferation. The proliferative response of PBLs to IL-2 peaked ≈1.5 days earlier than observed for ISG15-stimulated cultures (Fig. 2*A*). Thus, ISG15 appeared to either act on a different population of cells than IL-2 or act through intermediary molecules.

Mature ISG15 is derived from a 17-kDa precursor in the cytoplasm of IFN-α/β-treated cells (7). Eight amino acids are removed from the carboxyl terminus of pre-ISG15 to yield the mature ISG15 that is secreted. pre-ISG15 did not stimulate [^{3}H]thymidine incorporation by PBLs when compared to control cultures (Table 1). Thus, processing of pre-ISG15 to ISG15 may be a requisite for formation of biologically active ISG15.

Immunophenotyping of ISG15-Induced PBL Proliferation. PBL cultures were analyzed by flow cytometry after addition of ISG15 to identify the phenotype of the responding cells. There was an increase in the percentage of $CD56^+$ cells and a concomitant decrease in the percentage of $CD3^+$ cells in the cultures. When correlated with cell number, ISG15 induced a 26-fold increase in the number of $CD56^+$ cells as compared to the control culture after 8 days (Table 2). The number of $CD3^+$ cells in ISG15-treated cultures remained similar to untreated cultures. There was no significant change in the number of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells. Thus, ISG15 induced expansion of NK cells without significantly elevating the number of T cells.

Activation of Non-Major Histocompatibility Complex-Restricted Cytotoxicity by ISG15. Expansion of a $CD56^+$ population suggested that ISG15 may activate cytokine-dependent, NK/LAK cytotoxicity. To assess this, PBLs were incubated for 4 days with or without ISG15 and cocultured with ^{51}Cr-labeled target (Daudi or K562) cells in a 3-hr cell-mediated lympholysis assay. Treatment of PBL cultures with ISG15 resulted in significant induction of LAK-like activity [80 lytic units 30% (LU_{30}) per 10^6 cells] as evidenced by killing of Daudi tumor cell targets (Fig. 3). ISG15-activated PBL cultures also displayed increased lytic activity (100 LU_{30} per 10^6 cells) against K562 tumor cell targets. This enhanced lytic activity was primarily the result of increased LAK-like activity. Thus, ISG15 enhanced lytic activity of NK-derived LAK effector cells.

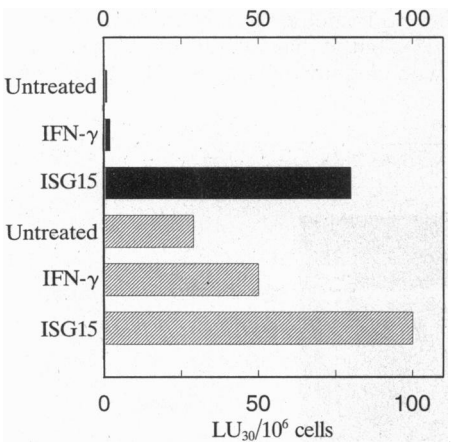

FIG. 3. Induction of LAK-like activity in ISG15-treated PBL cultures. PBLs were cultured for 4 days with ISG15 (100 ng/ml) or IFN-γ (10^3 units/ml) or were untreated. Cytolytic activity ($LU_{30}/10^6$ cells) in the cultures was measured against ^{51}Cr-labeled Daudi tumor targets (solid bars) or K562 tumor targets (hatched bars).

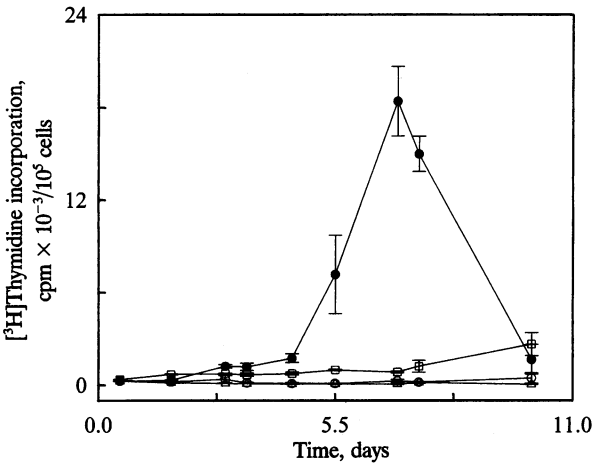

FIG. 4. Separated T and NK cells did not proliferate after ISG15 treatment. PBLs (10^6 cells per ml), immunomagnetically purified T cells (10^6 cells per ml), or immunomagnetically purified NK cells (10^5 cells per ml) were cultured with or without ISG15 (100 ng/ml). Proliferation was assessed at various time points by a 12-hr [^{3}H]thymidine incorporation. Groups were as follows: untreated PBLs (△), PBLs plus ISG15 (●), T cells plus ISG15 (□), NK cells plus ISG15 (○).

ISG15 Did Not Induce Proliferation of Separated T and NK Cells. To investigate whether ISG15 directly stimulated NK cells, proliferation of purified NK cells treated with ISG15 was assessed. PBLs were separated into purified T cells and NK cells by immunomagnetic methods. Cells were treated with or without ISG15 for various periods of time and radiolabeled for 12 hr with [^{3}H]thymidine. Neither purified T or NK cells proliferated in response to ISG15; however, PBLs proliferated after treatment with ISG15 (Fig. 4). Separation of T and NK cells did not render them refractory to stimuli since both populations responded to IL-2 treatment (Table 3). These results suggested that an intermediary molecule(s) or cell contact may be important for the NK cell response to ISG15.

ISG15 Induced IFN-γ from $CD3^+$ cells. Because purified NK cells did not respond to ISG15 and soluble factor(s) may have mediated ISG15-induced NK cell activation, the levels of IL-2 and IL-12, cytokines known to induce IFN-γ and directly stimulate NK cell activation (15–19), were measured. IFN-γ, but not IL-2 or IL-12, was detected by ELISA in ISG15-treated cultures (Table 4). Maximal accumulation of IFN-γ (>700

Table 3. Stimulation of separated T and NK cells with IL-2

Cells	Treatment	[^{3}H]Thymidine incorporation, cpm × 10^{-3} ± SEM per 10^5 cells
PBLs	None	0.1 ± 0.01
	IL-2	122 ± 3.5
T ($CD3^+$)	None	0.59 ± 0.1
	IL-2	66.8 ± 1.9
NK ($CD56^+$)	None	0.04 ± 0.02
	IL-2	105 ± 2.4

PBLs (10^6 cells per ml), immunomagnetically purified T cells (10^6 cells per ml), or immunomagnetically purified NK cells (10^5 cells per ml) were cultured with or without IL-2 (100 units/ml) for 5.5 days. Proliferation was assessed by a 12-hr [^{3}H]thymidine incorporation.

Table 4. ISG15 induced IFN-γ, but not IL-2 and IL-12, in PBL culture supernatants

Treatment	Cytokine measured	Culture time, hr 12	25.5	48	72
− ISG15	IFN-γ, unit/ml	<1	<1	<1	<1
	IL-2, pg/ml	<5	<5	<5	<5
	IL-12, pg/ml	<6	<6	<6	<6
+ ISG15	IFN-γ, units/ml	107	435	735	507
	IL-2, pg/ml	<5	<5	<5	<5
	IL-12, pg/ml	<6	<6	<6	<6

PBLs (10^7 cells per ml) were cultured with or without ISG15 (100 ng/ml), and cell-free supernatants were assayed for IFN-γ, IL-2, and IL-12.

units/ml) was measured 48 hr after addition of ISG15. This was consistent with data published previously in which medium from ISG15-treated cells was unable to sustain the proliferation of CTLL-2 cells, an IL-2-dependent cell line (9). Taken together, these results suggested that IL-2 or IL-12 did not mediate the biological effects of ISG15.

Both T cells and NK cells are sources of IFN-γ in response to appropriate stimulation. Because of the requirement for T cell and NK cell coculturing for ISG15-induced NK cell proliferation, it suggested that ISG15 may not stimulate IFN-γ production from NK cells. To identify which cell type produced IFN-γ in response to ISG15, $CD3^+$ cells were separated from $CD56^+$ cells and independently treated with ISG15. RT-PCR analysis with IFN-γ-specific primers demonstrated induction of IFN-γ mRNA in PBLs ($CD3^+$ and $CD56^+$ mixed populations) and purified $CD3^+$ cells cultured with ISG15 (Fig. 5). Purified $CD56^+$ cells did not synthesize IFN-γ mRNA after treatment with ISG15 (Fig. 5). The band corresponding to IFN-γ mRNA was more intense in the PBLs plus ISG15 lane than in the $CD3^+$ plus ISG15 lane (Fig. 5). RT-PCR was not quantitative. Repeated experiments confirmed the qualitative result but did not always confirm the more intense band in the PBLs plus ISG15 lane. Thus, analysis of IFN-γ mRNA expression suggested that the high levels of IFN-γ induced by ISG15 in PBL cultures was a product of T cells.

Proliferation of $CD56^+$ cells and potent induction of LAK cell activity by ISG15 was not the result of IFN-γ production alone from $CD3^+$ cells, since recombinant IFN-γ failed to induce PBL proliferation (Table 1) and did not enhance induction of LAK-like activity (< 2 LU_{30} per 10^6 cells) (Fig. 3).

DISCUSSION

ISG15 induced the production of IFN-γ, proliferation of NK cells, and formation of NK-derived LAK cells in cultures of B-depleted lymphocytes. Numerous lines of evidence support the conclusion that modulation of immunological function was due to recombinant ISG15 and not a bacterial contaminant. Recombinant ISG15 was purified to homogeneity as assessed by Coomassie blue staining of SDS/polyacrylamide gels (data not shown). Heat denaturation of biologically active ISG15 did not induce proliferation, and biological activity was neutralized by addition of polyclonal antibodies raised against native ISG15. An affinity-purified antibody raised against recombinant ISG15 blocked proliferation (data not shown). Lipopolysaccharide levels were <0.03 EU/μg of protein, and addition of lipopolysaccharide to PBL cultures did not stimulate proliferation (data not shown). Murine ISG15 and pre-ISG15 failed to stimulate proliferation, suggesting that a copurifying bacterial protein was not responsible for biological activity. In addition, human ISG15 did not stimulate the proliferation of B-depleted murine lymphocytes, indicating that the biological activity was species-specific (N. Garlie, J.D., E.C.B., and A.L.H., unpublished observations). Finally, ISG15 purified from IFN-treated Daudi cells stimulated the production of IFN-γ (E.K., unpublished observations).

Since the beginning of studies of ISG15 biological activity (9), progress has been hindered by our inability to consistently purify recombinant ISG15 as a biologically active molecule. This is likely due to improperly folded protein. The existence of non-native ISG15 has been supported by inconsistent CD spectra that fail to converge to a unique solution (11), cleavage of the carboxyl-terminal glycine dipeptide during purification (J. Narasimhan and A.L.H., unpublished results), and aggregation resulting in part from disulfide dimerization via the single cysteine residue (J. Narasimhan and A.L.H., unpublished results).

ISG15 induced NK cell expansion and the formation of NK-derived LAK cells. This was not a direct effect of ISG15 on the NK cell, but rather required coculturing of T and NK cells. NK cell proliferation occurred in the absence of detectable levels of IL-2 or IL-12, but ISG15 was a potent inducer of IFN-γ. Although both T cells and NK cells are sources of IFN-γ in response to other cytokines and stimuli (15, 16, 20, 21), ISG15 induced IFN-γ production from only T cells. Lack of IFN-γ mRNA synthesis in NK cells was consistent with a lack of direct effect of ISG15 on NK cell activation and proliferation. Lack of induction of IFN-γ in NK cells was consistent with bioassays in which culture supernatants from ISG15-activated $CD3^+$ cells, but not $CD56^+$ cells, activated macrophages (9). It is not possible to absolutely exclude that ISG15-treated NK cells did not make any IFN-γ, but no mRNA was detected in NK cells using RT-PCR, and purified NK cells did not proliferate when treated with ISG15.

ISG15 has no homology to any known secreted protein or cytokine (22), but it has significant amino acid sequence homology with ubiquitin (11), an 8.6-kDa intracellular protein

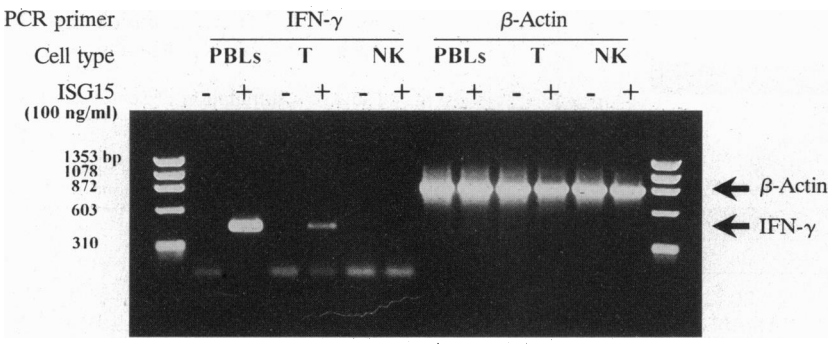

FIG. 5. Influence of ISG15 on the synthesis of IFN-γ mRNA in $CD3^+$ and $CD56^+$ cells. PBLs were immunomagnetically separated into T and NK cell populations. PBLs (10^7 cells per ml), T cells (10^7 cells per ml), and NK cells (10^6 cells per ml) were cultured with (+) or without (−) ISG15 (100 ng/ml). T and NK cells were cultured at different densities to approximate their concentrations in PBLs. After 12 hr of culture, total RNA was isolated, and RT-PCR was performed with IFN-γ-specific or β-actin-specific primers. PCR reactions were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide fluorescence.

that is covalently ligated via its carboxyl terminus to proteins, thereby targeting them for degradation (23, 24). Early investigations into the biological function of ubiquitin identified it as a molecule that induced differentiation of T lymphocytes and B lymphocytes (25, 26). In our system, ubiquitin did not enhance [^{3}H]thymidine incorporation by PBLs (Table 1).

The lack of biological activity of pre-ISG15 suggested that removal of carboxyl-terminal amino acids from pre-ISG15 may be important for biological activity. Lack of biological activity of pre-ISG15 was not likely due to misfolded pre-ISG15 because the converting enzyme correctly processed pre-ISG15 to mature ISG15. Further, heat-denatured pre-ISG15 was not converted to mature ISG15 (J. Rasmussen and A.L.H., unpublished results).

The cytokine network is composed of many molecules with overlapping and complementary functions. The *in vivo* role of each molecule is likely linked to specific steps in immunological activation. ISG15 may represent an important mediator of the host response to IFN-α/β by amplifying the immunomodulatory effects of IFN-α/β. During the host response to infection by virus, IFN-α/β are produced from the infected cell, which may induce the synthesis of ISG15 in the infected cell or neighboring cells. Once synthesized, ISG15 can be secreted or released by lysis of the infected cell. It may then induce the production of IFN-γ from T cells, augment NK cell proliferation, activate non-major histocompatibility complex-restricted cytolytic lymphocytes, and activate monocytes and macrophages via the induced IFN-γ. The selective induction of IFN-γ from T cells is believed to be important in regulating the immune response.

These studies have defined ISG15 as a unique member of the cytokine cascade that may be responsible for some of the immunomodulatory effects of IFN-α/β. Understanding ISG15's biological properties should aid in dissecting the host response to intracellular pathogens and tumors, potentially leading to its use as an immunotherapeutic agent.

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