REVERSAL OF INFECTIOUS MONONUCLEOSIS-ASSOCIATED SUPPRESSOR T CELL ACTIVITY BY D-MANNOSE

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The molecular basis for selective interaction among different cells of the immune system is not well understood. Recent work has indicated that specific cell surface carbohydrates may serve as recognition and interaction structures. It has been shown that sugar molecules can provide exquisite specificity to cell surface structures such as blood group determinants (1–3); may be necessary for cell-cell contact required for fertilization (4, 5), differentiation (6, 7), cell aggregation (8, 9), and infection with viruses and bacteria (10, 11); may be involved in the interaction between natural killer cells (12, 13), cytotoxic T cells (14), and suppressor T cells (15) with their targets; and provide receptors required for cell activation by mitogens (16). Elegant studies on the survival of glycoproteins in the circulation have clearly demonstrated that this is dependent upon the nature of the terminal sugar residue on the molecule. For example, it has been shown that uptake of galactose-terminated glycoproteins by hepatocytes involves recognition and binding of the terminal sugar molecules to a lectin-like receptor on the liver cell surface (17). Similar findings were reported for the uptake of D-mannose, N-acetyl-D-glucosamine and L-fucose-terminated glycoproteins by cells of the reticuloendothelial system (18, 19).

Sugars have an enormous potential for structural diversity that makes them excellent carriers of biological information. Unlike peptides and oligonucleotides, which depend only on the sequence and number of different monomeric units to encode information, sugar polymers have additional potential diversity because of multiple forms of linkage of the glycosidic units and the possibility of forming branched structures. In this report, we have investigated the nature of signals operating in the interaction between suppressor T cells and their targets and have explored the role of sugars in immunoregulation. We have used T cells obtained from the peripheral blood of patients with acute Epstein-Barr virus (EBV)1-induced infectious mononucleosis (IM) as a source of suppressor T lymphocytes, since during this illness suppressor T cells become activated that profoundly inhibit immunoglobulin (Ig) production in vitro (20, 21). Our results demonstrate that D-mannose and some of its derivatives can significantly reverse suppression by the T cells and suggest an important role for certain sugars as recognition and regulatory signals for the immune system.

1Abbreviations used in this paper: AET, 2-aminoethylisothiouronium bromide; αMM, α-methyl-D-mannoside; D-Man, D-mannose; EBV, Epstein-Barr virus; IgSC, Ig-secreting cells; IM, infectious mononucleosis; MNC, mononuclear cells; PWM, pokeweed mitogen; SISS, soluble immune suppressor substance; SRBC, sheep erythrocytes.
Materials and Methods

Peripheral Blood Leukocytes. Heparinized blood was obtained from patients with acute EBV-induced IM and normals. The diagnosis of EBV-induced acute IM was confirmed in each patient by the presence of IgM antibodies to the viral capsid antigen (22). Mononuclear cells (MNC) were obtained by density gradient centrifugation through Ficoll-Hypaque (LSM solution; Litton Bionetics Inc., Kensington, MD). Cell subsets enriched for B and T cells were obtained as previously described (23) by incubating the MNC for 2 h at 4°C at a ratio of 1:150 with 2-aminoethylisothiouronium bromide-treated sheep erythrocytes (AET-SRBC), and separating the rosette-forming cells on a Ficoll-Hypaque gradient. Nonrosetting MNC containing <2% T cells and a variable number of surface Ig-positive cells (20-50%) will be referred to as the "B cell population." The pellet of rosetted MNC was treated with ammonium-chloride lysing buffer to remove SRBC. This cell suspension, containing <2% nonrosetting cells will be referred to as the "T cell population."

Lymphocyte Cultures and Assay for Ig Production and Proliferation. All cell populations were washed three times in Hanks' balanced salt solution (B and B Research Laboratories Inc., Baltimore, MD) and resuspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) containing 2 mM glutamine (Gibco Laboratories), 5 µg/ml gentamycin (Sigma Chemical Co., St. Louis, MO), and 10% heat-inactivated fetal bovine serum (Reheis Chemical Co., Phoenix, AZ). The cells were cultured in 1 ml vol, at various concentrations in 16 x 125-mm round-bottomed tubes (Falcon 3053; Falcon Labware, Oxnard, CA) at 37°C in a 5% CO 2 humidified incubator with or without the addition of pokeweed-mitogen (PWM) (Gibco Laboratories) at a final concentration of 5 µl/ml or EBV (filtered supernatant of the B95-8 lymphoblastoid cell line containing ~10^6 transforming U/ml) at a final dilution of 1:10. All sugars (Sigma Chemical Co.) were prepared as 250 mM stock solutions in RPMI 1640 medium, filtered, and stored at 4°C. Purified D-mannose and α-methyl-D-mannoside were a kind gift of Dr. Ulrich H. Koszinowski, Federal Research Institute for Animal Virus Disease, Tubingen, Federal Republic of Germany. Yeast mannan (Sigma Chemical Co.) was prepared as a 1 mg/ml stock solution in RPMI 1640 medium. At termination of the culture period, the cells were washed three times in balanced salt solution and the number of Ig-secreting cells (IgSC) was determined, in duplicate, with a reverse hemolytic plaque assay, as previously described (23). Plaque reading was performed with a video-based semiautomated plaque counter (Optomax, Hollis, NH) and the IgSC response in each group was expressed as the geometric mean.

For proliferation assays, 2 x 10^5 T cells were cultured for 5 d in a total volume of 0.2 ml, in triplicate, in RPMI 1640 media, supplemented with 10% fresh normal human plasma, 2 mM glutamine, and 5 µg/ml gentamycin, in flat-bottomed microtiter plates (Falcon Labware). [3H]Thymidine (NET 027; New England Nuclear, Boston, MA) (specific activity 6.7 Ci/mM, final concentration 0.5 Ci per culture) was added during the final 4.5 h of culture and the plates then harvested. Results of liquid scintillation counting were calculated as mean counts per minute of triplicate cultures. The proliferative response in each group was expressed as the geometric mean.

Results

A Role for Sugars in Suppressor Cell/Target Interactions. Acute EBV-induced IM is associated with the appearance in the peripheral blood of activated suppressor T lymphocytes that profoundly inhibit B cell activation in vitro (20, 21). Suppression by IM T lymphocytes can be easily demonstrated in vitro by comparing the number of IgSC produced by PWM-stimulated normal MNC when cultured alone, with that generated in co-cultures containing the same PWM-activated normal MNC together with IM T cells. In preliminary experiments we examined the effects of a panel of various sugars on IM suppressor T cell activity. As shown in Table I, whereas most of the sugars tested had no effect, D-mannose (D-Man)
and three mannose-related sugars, \(\alpha\)-methyl-D-mannoside (\(\alpha\)MM), mannose-6-phosphate, and mannan, substantially reversed IM T cell suppression. Other mannose-related monosaccharides, however, had no effect, nor did L-mannose, the stereoisomer of the active sugar D-mannose.

To determine whether certain sugars consistently inhibited suppressor T cell activity in this system, similar experiments were performed using T cells derived from 12 patients with acute IM (Fig. 1). T cells from the IM patients suppressed the normal MNC response to PWM by 75% in these experiments. The addition of 25 mM D-Man or \(\alpha\)MM to the normal PWM-stimulated indicator cells had no significant effect on the response of these cells, but addition of these same sugars to the suppressed co-cultures resulted in a marked restoration of the IgSC response. At least three additional monosaccharides, including \(N\)-acetyl-D-glucosamine, L-rhamnose, and gentiobiose were used in each of these experiments (data not shown), and they demonstrated no significant effect on Ig production by either normal cells or co-cultures of normal and patients' T cells. Thus, D-Man and \(\alpha\)MM consistently reversed suppression mediated by IM T lymphocytes, but had little or no effect on Ig production by normal MNC, indicating that these sugars were affecting the process of suppression, rather than directly stimulating the responder MNC cells.

Suppressor T lymphocytes activated during acute IM are known to suppress
autologous, in addition to allogeneic, B cell responses (23). We wished to assess whether D-Man and αMM were effective in reversing suppression of autologous combinations of IM B and T cells stimulated with EBV. In four separate experiments, \(0.5 \times 10^6\) B cells were cultured with EBV, either alone or in the presence of autologous T cells (\(2.0 \times 10^6\)), and Ig production was determined after 10 d in culture. EBV-activated B cells produced a mean of 5,913 (SEM, 1.12) IgSC and cultures containing mixtures of autologous EBV-infected B and T cells generated a mean of 455 (SEM, 1.67) IgSC, indicating a profound T cell suppression (93% inhibition). In contrast, identical B and T cell mixes produced 3,692 (SEM, 1.22) IgSC in the presence of 25 mM D-Man, and 4,094 (SEM, 1.29) IgSC in the presence of 25 mM αMM. These findings demonstrate that D-Man and αMM can reverse suppression in autologous as well as allogeneic cell combinations, when either a T helper cell-dependent or -independent activator was used as the B cell stimulant.

Dose-Response Relationships for Sugar Inhibition and Quantitation of the Effect on
Suppression. Fig. 2 illustrates the effect of various concentrations of d-Man and αMM on the suppression of Ig production by IM T cells. At concentrations of 3 mM or less, neither sugar had a detectable effect on the suppressor activity. At 6.25, 12.5, and 25 mM, an increasing degree of reversal of suppression was observed. At concentrations >25 mM, nonspecific toxic effects were noted with a diminished response of the PWM-stimulated indicator cells (not shown); therefore 25 mM was the highest concentration routinely tested in these studies.

In an attempt to partially quantitate the effect of the sugars on IM suppressor T cell activity, studies were done to compare the number of T cells required to achieve a given level of suppression in the absence of added monosaccharide with the number of cells required to achieve the same degree of suppression in the presence of 25 mM αMM. Fig. 3 summarizes the results of seven separate experiments and shows that ~5 x 10⁵ IM T cells in regular media suppressed Ig production to a similar level as 2 x 10⁶ of these same IM T cells in medium containing 25 mM αMM. Thus, fourfold more suppressor T cells were required in the presence of this sugar.

Mode of Action of Sugars in Reversing Suppression. d-Man has been shown to inhibit T cell proliferation to stimulation with soluble antigens (24), so that inhibition of suppressor function could be related to this antiproliferative effect.
on T cells. To test this possibility, PWM-stimulated proliferation of T cells from normal and IM patients was studied in the presence of αMM and mannose-6-phosphate as well as N-acetyl-D-glucosamine. As shown in Table II, although D-Man inhibited both normal and IM T cell proliferation to PWM, mannose-6-phosphate, a sugar also capable of reversing suppression by IM T cells, was not inhibitory. Thus, reversal of suppression by these monosaccharides did not necessarily depend on an antiproliferative effect of the sugar on the T cell population. It is interesting that the sugars that inhibited the T cell suppression of B cell responses did not detectably augment the proliferative response of the T cells from these patients. If T cell suppression is the mechanism of decreased proliferative responses to PWM in acute IM (25), this suppressor mechanism is not reversed by the sugars tested.

### Table II

| Additives                        | Normal T cells | IM T cells |
|---------------------------------|----------------|------------|
| Media                           | 399 (1.29)     | 154 (1.35) |
| PWM                             | 24,439 (1.34)  | 3,821 (1.15)|
| PWM + N-acetyl-D-glucosamine    | 13,788 (1.12)  | 2,630 (1.38)|
| PWM + α-mannose                 | 11,245 (1.38)  | 2,047 (1.19)|
| PWM + α-methyl-D-Mannoside      | 24,658 (1.16)  | 3,033 (1.33)|
| PWM + Mannose-6-phosphate       | 30,793 (1.18)  | 3,727 (1.38)|

* Concentration is expressed as cpm (SEM) (four determinations).

† 5-d cultures of 200,000 normal and IM T cells.
The lack of a direct enhancing effect of D-Man and αMM on Ig production by normal MNC and the lack of correlation between the antiproliferative effect and the effect of suppression suggested that these sugars were reversing suppression by interfering in the interaction between the regulatory T cells and the responder B cells. To investigate at which point in the process of suppression these sugars were acting, we added D-Man and αMM at different intervals after initiation of the cultures. In a typical experiment, PWM-activated normal MNC (0.5 × 10⁶) produced 12,540 IgSC when cultured alone and 1,660 IgSC in co-culture with IM T cells (0.5 × 10⁶); identical PWM-activated co-cultures of normal MNC and IM T cells produced 3,940 IgSC when D-Man was added at the initiation of the cultures, and 1,640 IgSC when D-Man was added only 24 h after culture establishment. This demonstrated that the sugars had to be present early in culture to be effective, and suggested that the antiinhibitory effect of sugars depended upon their interference with early cell interactions that lead to suppression, rather than their enabling the B cells to escape suppression after this had been established. Interestingly, we had previously shown that IM suppressor T cells exert their principal inhibitory effect during the first 24 h of culture (21).

The ability of D-Man and some of its derivatives to interfere with suppression by regulatory IM T cells could be due to a direct effect of these sugars on the suppressor T cells or to an effect on the normal responder B cell, by making them nonsuppressable. Alternatively, these sugars might not directly affect either the responder or the regulatory cells, but could be acting upon an "inhibitory soluble mediator" produced by the suppressor T lymphocytes or preventing necessary direct cell-cell interactions between the T and B cells. To investigate some of these possibilities, we separately preincubated normal MNC and IM T cells for 4–20 h in the presence of either 50 mM αMM or media alone at 37°C on a rocker platform. After incubation, the cells were washed and set up in culture for 7 d in different combinations with PWM. We reasoned that if the reversal of suppression was due to an early effect of the sugar on either responder or suppressor cell population, this could be detected in co-cultures where one but not the other cell population had been exposed to the sugar. The results of these experiments are shown in Fig. 4. Preincubation of either normal MNC or IM suppressor T cells with αMM had little or no effect on either the ability of normal MNC to be suppressed, or on the ability of IM T cells to inhibit the IgSC response by normal PWM-stimulated MNC. In addition, suppression of Ig production was detected in cultures containing normal cells and regulatory T cells that had both been separately preincubated with αMM. Furthermore, the observed suppression was significantly reversed when αMM was present during the 7-d co-cultures, whether or not IM T cells and/or normal MNC had been preincubated in medium containing 50 mM αMM. These findings suggested that the inhibition of suppression by monosaccharides was not due to an irreversible effect of the sugar on either suppressor or responder cells. The observation that IM T cells preincubated in αMM were as inhibitory as those preincubated in media indicated that this sugar was not reversing suppression because of a selective, acute toxicity on the inhibitory T cells. In addition, these results suggested that either the interaction between the monosaccharide and the cell providing the specific sugar receptor was of low affinity and readily reversible,
FIGURE 4. Normal MNC and IM T cells were precultured in medium alone or medium containing 50 mM αMM for 4–20 h at 37°C, washed, and then cultured for 7 d with PWM. Normal MNC were cultured either alone (0.5 × 10^6) or with IM T cells (0.5 × 10^6), with or without the addition of 25 mM αMM. Mononuclear cells preincubated in media containing 50 mM αMM are indicated as MNC (αMM); IM T cells preincubated with αMM are indicated as IM T (αMM). Not shown: In the presence of 25 mM αMM, co-cultures of normal MNC and IM T cells produced 5,930 IgSC when the T cells had been preincubated with αMM, 5,710 IgSC when the normal MNC had been preincubated with αMM, and 5,060 IgSC when both normal MNC and IM T cells had been separately preincubated with αMM.

or that the sugar interfered with the effects of a soluble mediator rather than by acting directly on the cells. We were, however, unable to detect any suppressor factor activity in culture supernatants of IM T cells, IM B and T cells, with or without PWM or EBV, or sonicates of IM T cells. Thus we were unable to find any evidence supporting a role for soluble suppressor mediators in this process.

Discussion

EBV-induced IM is a particularly interesting disease from an immunologic standpoint. Relatively distinctive features of this illness include T cell lymphocytosis and polyclonal hypergammaglobulinemia. It is believed that EBV is directly responsible for the appearance of increased numbers of IgSC in the circulation (21) and that hypergammaglobulinemia in IM is the result of an in vivo polyclonal activation of the B cells by this virus (21, 26). Recent studies have further characterized the lymphocytosis as consisting of T cells that belong to the T8-positive subclass which includes suppressor/cytotoxic T lymphocytes (25, 27), and functional analysis has revealed that they usually inhibit B cell activation in vitro (20, 21). Suppressor T lymphocytes in acute IM are not specific for EBV-induced activation, since PWM-induced Ig production is also markedly suppressed (20, 21). In addition, they are not restricted by the major histocompati-
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bility complex, since they inhibit responses by allogeneic as well as autologous lymphocytes (20, 21, 23).

In this study, we have attempted to analyze the mechanism by which IM suppressor T cells recognize and inhibit B cell activation in vitro. A number of investigators have examined the possibility that sugars might be involved as recognition signals in immune interactions. It was reported that Ia antigens are rich in carbohydrates and that binding of allogeneic and xenogeneic anti-Ia antibodies to Ia antigens can be inhibited by certain sugars (28). Studies on natural cytotoxicity and natural killer cells showed that these can be prevented from lysing allogeneic target cells by D-Man in the mouse, and in the human by mannose-6-phosphate, fructose-1-phosphate, and fructose-6-phosphate (12, 13). The lysis and, presumably, the recognition of different target erythrocytes by cytotoxic mononuclear phagocytes isolated from invertebrates such as the starfish and keyhole limpet is inhibited by the addition of various oligosaccharides, each target showing a different profile of inhibitory sugars (29). Similar studies on cytotoxic T cells generated in mixed lymphocyte reactions in the mouse showed that they could be prevented from killing by the addition of 2-deoxy-d-glucose (14), and that mouse suppressor T cells, generated by a 6-d in vitro preculture, could be counteracted by the addition of aMM in secondary cultures (15). Also, it was reported that the effects of soluble mediators, such as migration inhibitory factor (MIF), soluble immune suppressor substance for B cells (SISS-B), soluble immune suppressor substance for T cells (SISS-T), and continuous T cell line soluble immune suppressor substance (CTC SISS-B) could be inhibited by specific sugars, including L-rhamnose, L-fucose and N-acetyl-d-glucosamine (30–32).

In view of these studies, we have hypothesized that the interaction between suppressor T cells, activated during IM, and their target B cells might depend upon the presence of a lectin-like structure on one cell surface and a specific carbohydrate molecule on the other interacting cell. If this was the case, the addition of large quantities of a specific sugar to the suppressed cultures could competitively saturate the lectin's binding capacity, and thus could interfere with suppression. We have shown that D-Man and some of its derivatives, including aMM, mannose-6-phosphate, and mannan, significantly enhanced Ig production by suppressed cultures, while many other sugars did not. Since D-Man and aMM had little or no effect on Ig production by normal cells, and enhancement of Ig production by these sugars only occurred when the normal cells were co-cultured with inhibitory T cells, we concluded that D-Man and aMM were interfering with the process of suppression.

The mechanism by which these sugars reverse suppression is unclear at present. Our data show that the reversal of suppression could not be attributed to toxicity of D-Man and derivatives for the regulatory T cells, since preincubation of these cells with the sugars did not decrease their ability to suppress when washed free of sugar. Also, mannose-6-phosphate, at a concentration used to reverse suppression, had little or no effect on the ability of T cells to respond to mitogens with proliferation. Furthermore, the inability of these sugars to reverse suppression when added 24 h after the cultures had been initiated suggested that the sugars act at a very early stage in the interaction between suppressor and responder cells, rather than providing the B cell a way to escape suppression.
after this process has been initiated.

We have addressed the question of which cell type bears the mannose-specific lectin-like receptor by preincubating either the suppressor T cells or the normal responder cells in the presence of D-Man. One might expect that this sugar would bind to its specific lectin on the cell surface that expressed it, and, as a consequence, would prevent either the suppressor T cells from suppressing or the responder cells from being suppressed. Preincubation with D-Man or α-MM, however, was ineffective and neither prevented the regulatory T cells from suppressing nor made the responder mononuclear cells unresponsive to inhibition by IM T cells. This suggested that if a mannose-specific lectin-like structure was present on these cells, either the binding affinity for the specific sugars tested (presented as soluble monosaccharides) was low, or the sugar was rapidly internalized and the specific receptor continuously regenerated. A low binding affinity might be expected since it is unlikely that the cellular receptor consists solely of a single D-Man molecule. Furthermore, the relatively high concentration of soluble mannose required to reverse suppression is consistent with inhibition due to a cross-reaction between D-Man and the true receptor structure. Alternatively, instead of a cell surface, a soluble factor-mediating suppression might have provided the sugar-binding component, even though we were consistently unable to demonstrate the presence of inhibitory factors in the supernatant of our suppressed cultures or in extracts of IM T cells. Thus our studies have failed to determine which structure bears the mannose-specific receptor, but clearly indicate that D-Man and some of its derivatives can consistently counteract suppression by IM T lymphocytes in vitro. Since activation of suppressor T lymphocytes in IM probably represents an important mechanism of defense for this viral infection, our studies suggest that certain sugars have an important role in physiologic immune cell interactions.

Summary

Epstein-Barr virus-induced infectious mononucleosis (IM) is associated with the activation of suppressor T lymphocytes that profoundly inhibit immunoglobulin (Ig) production in vitro. We have examined the nature of signals operating in the interaction between IM suppressor T cells and their targets, and explored the possibility that a lectin-like receptor molecule and its specific sugar might provide specificity to this interaction. When D-mannose or some of its derivatives, including α-methyl-D-mannoside, mannose-6-phosphate, and mannan, were added to suppressed cultures containing IM T lymphocytes and pokeweed mitogen (PWM)-stimulated normal mononuclear cells, a significant enhancement of Ig production was observed. These sugars had little or no effect on Ig production by the PWM-stimulated responder cells alone and thus the enhanced Ig production could be attributed to the reversal of suppression in the co-cultures by these sugars. This was further confirmed by the observation that the sugars were effective only if present during the first 24 h of culture, a time when IM suppressor T cells exert their principal effect. The effect of sugars on Ig production by suppressed cultures was similar to that achieved by decreasing by about fourfold the number of IM T cells in culture. The effect of the sugars is unlikely to represent a form of nonspecific toxicity, since inhibited cultures
become responders in the presence of the sugar. Furthermore, toxicity restricted to the suppressor T cells is unlikely, since preincubation of the T cells with the sugars did not reduce their subsequent ability to suppress in secondary indicator cultures. In addition, there was no correlation between the effect of the sugars on T cell proliferation and their effect on T cell-mediated suppression. The reversal of suppression by sugars was dose dependent and demonstrated stereospecificity in that L-mannose was without effect while D-mannose reversed suppression. These data indicate that D-mannose and some of its derivatives consistently reverse suppression of Ig production by IM T cells and strongly suggest a role for saccharides as critical components in the cellular receptors involved in certain physiologic immune cell interactions.

We are indebted to Linda Wood and all the nursing staff of Georgetown University Student Health Center for their help in obtaining the blood specimens and to Dr. W. Henle of Children’s Hospital in Philadelphia for his help in determining the anti-EBV antibody titers in our patients.

Received for publication 9 May 1983 and in revised form 27 June 1983.

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