A New Approach to the Oral Administration of Insulin and Other Peptide Drugs

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The oral administration of peptide drugs is well known to be precluded by their digestion in the stomach and small intestine. As a new approach to oral delivery, peptide drugs were coated with polymers cross-linked with azoaromatic groups to form an impervious film to protect orally administered drugs from digestion in the stomach and small intestine. When the azopolymer-coated drug reached the large intestine, the indigenous microflora reduced the azo bonds, broke the cross-links, and degraded the polymer film, thereby releasing the drug into the lumen of the colon for local action or for absorption. The ability of the azopolymer coating to protect and deliver orally administered peptide drugs was demonstrated in rats with the peptide hormones vasopressin and insulin.

THE ADVANTAGES TO MILLIONS OF diabetics of oral administration of insulin are so great that much effort was directed just after the discovery of insulin by Banting and Best to the development of alternatives to injection (*1, 2*), but without success. Two apparent barriers to oral administration of insulin are (i) the inactivation of insulin, a 51–amino acid polypeptide, by digestion in the gastrointestinal tract, and (ii) the lack of knowledge of a mechanism to transport peptides with more than three amino acid residues from the gut to the blood. More recently, buccal (*3*), nasal (*4*), and rectal (*5*) administration of insulin have been used successfully to lower blood glucose and even to control diabetes. Despite its size, insulin crosses intestinal (*6*), respiratory (*7*), and oral mucosa (*3*) in normal and diabetic subjects. Although the efficacy is lower than by injection of insulin, the results are encouraging enough to warrant a reexamination of oral administration.

Peptide drugs can be protected against attack by digestive enzymes by coating the drugs with an impermeable polymer film. The coating polymer must not be susceptible to the action of digestive enzymes. If the coating is cross-linked and the cross-link is broken by microflora normally present in the colon, then the coating will degrade in the colon and the drug will wash out into a part of the intestine devoid of secreted digestive enzymes. The drug can then act locally in the colon, or be available for possible absorption into the blood. A covalent functionality susceptible to cleavage by bacterial action is the azoaromatic group $R–C_6H_4–N=N–C_6H_4–R$, which can be cleaved by reduction to form a pair of aromatic amines, $R–C_6H_4–NH_2 + H_2N–C_6H_4–R$. The ability of microflora to reductively cleave azoaromatic bonds has been known since 1937, when Brohm and Frohwein (*8*) observed that bacteria reduced azo food colorings. In 1972 Peppercorn and Goldman (*9*) observed that salicylazosulfapyridine (Azulfidine, Sulphasalazine) (SAS), a drug used extensively in the management of ulcerative colitis, is cleaved by microflora in the gut to its components, aminosalicylic acid and sulfapyridine. More recently, Parkinson and co-workers (*10*) synthesized high molecular weight polymers bound to 5-aminosalicylic acid by aromatic azo groups as vehicles for the delivery of 5-aminosalicylic acid to the colon.

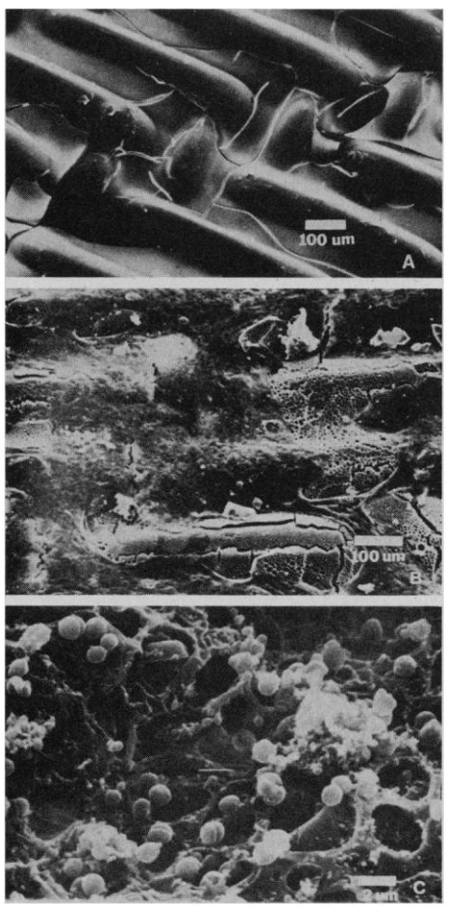

Fig. 1. Scanning electron micrographs of a film of a copolymer of styrene and hydroxyethylmethacrylate cross-linked with divinylazobenzene before (A) and after (B, low magnification; C, high magnification) anaerobic incubation with a culture of freshly voided human fecal material for 8 days. White spheres in C are adhering bacteria.

As a trial of the protective coating approach, copolymers of styrene and hydroxyethylmethacrylate (either 1:6 or 100% hydroxyethylmethacrylate), cross-linked with 1 or 2% divinylazobenzene or substituted divinylazobenzenes, were prepared by conventional methods (*11*). The products were purified either by prolonged washing to remove unreacted monomers, or by repeated reprecipitation from chloroform solution by the addition of hexane. The azoaromatic polymers dissolve in solvents such as methylene chloride or chloroform containing about 20% methanol by volume. The solution evaporates to form a tough, water-impervious film to coat the surface of paper strips, tablets, or gelatin capsules.

Several tests were carried out in vitro to demonstrate the reduction and degradation of the azoaromatic polymer by colonic microflora. Circular glass cover slips of 1-cm^2 area were coated with a 5- to 10-mg film by evaporation of a solution of azoaromatic polymer. These were incubated anaerobically for 3 to 8 days with shaking in Schaedler's medium inoculated with freshly voided human feces (*12*). The polymer samples were washed with distilled water, air dried, and reweighed. The azoaromatic polymer was dissolved in 5 ml of a 1:4 mixture of methanol and chloroform and the absorption spectrum was compared with that of the unincubated azoaromatic polymer. The absorbance at the λ_{max} (360 nm) decreased by more than 80%. Because the reduction of the azo cross-links does not yield water-soluble products, little or no change in the weight of the polymer was observed after incubation. To visualize the effect of microfloral action on the azoaromatic polymer, 16-mesh grids of nickel wire (1 cm square) were coated with azoaromatic polymer by dipping into a solution of the polymer in methanol-chloroform and drying. The coated grids were incubated with human fecal microflora as above. Incubated and unincu-

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bated grids were examined in the scanning electron microscope (Fig. 1). The unincubated grids showed a relatively uninterrupted surface. After incubation, the coating on the grids was pock-marked with holes, and a higher magnification revealed an irregular network of polymeric fibers replacing the film. In another test, azoaromatic polymer threads were incubated in anaerobic cultures of human fecal microflora and the elongation with a 2-g load was measured over 11 days of incubation. Threads in uninoculated medium elongated by a mean of only 12.5% in the first 3 days as the polymer absorbed some water, but threads exposed to bacteria continued to elongate at a faster rate, reaching two to almost four times their original length (Fig. 2).

Samples of an azo-cross-linked copolymer of styrene and hydroxyethylmethacrylate (1:6) were examined by the Ames test for toxicity and mutagenicity (*13*). There was no evidence of either toxicity or mutagenicity at the highest concentration tested (100 μg per plate).

Azoaromatic polymer-coated capsules or pellets of vasopressin and insulin were used to determine whether the azoaromatic polymer could be used for the oral delivery of peptide drugs. Natural lysine and arginine vasopressin, and the synthetic analog 1-deamino-8-D-arginine vasopressin (DDAVP), were diluted with powdered sucrose and loaded into gelatin Minicaps® (*14*), which were then coated with the azoaromatic cross-linked polymer. The coated capsules were deposited in the stomach of rats with a plastic tube and a stylet.

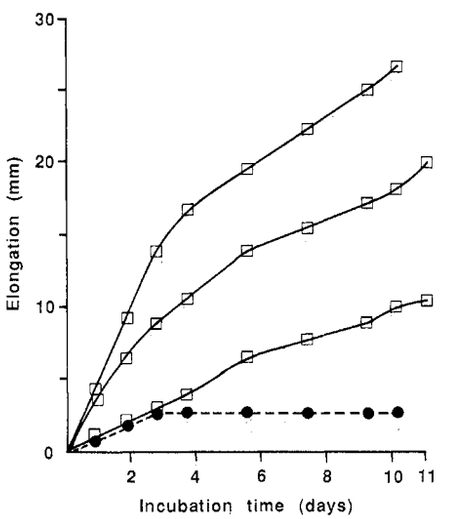

Fig. 2. Increase in length of 10-mm threads of azoaromatic cross-linked hydroxyethylmethacrylate polymer during anaerobic incubation in medium with (open squares) and without inoculation (filled circles) with human feces. The uninoculated curve represents the mean of three threads. The upper curves were from three other threads of the same polymer, incubated in inoculated medium. The threads were selected at random from a lot that varied in diameter from 0.07 to 0.25 mm. Because of variation in the thread thickness and the density of the inoculum, the results with the threads in the inoculated medium are not combined.

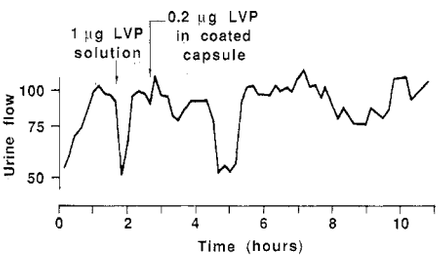

Fig. 3. Plot of urine output (drops per 10-minute period) by an ethanol-anesthetized, water-loaded female Sprague-Dawley rat given a dose of 1 μg (about 3 nmol/kg body weight) of lysine vasopressin (LVP) in 0.5 ml water, and 0.2 μg (0.6 nmol/kg) in powdered sucrose diluent in an azoaromatic polymer-coated gelatin capsule. The absorption of vasopressin is marked by a sudden fall in the rate of production of urine. This occurred about 2 minutes after the solution of vasopressin was given, and lasted for about 30 minutes; and occurred about 100 minutes after the vasopressin in the azoaromatic polymer coated capsule was given and lasted for about 60 minutes.

The biological test for the oral absorption of vasopressin was adapted (*15*) from Sawyer (*16*). A fasted, hydrated, and ethanol-anesthetized rat was given lysine vasopressin in solution and then in an azoaromatic polymer-coated capsule. In a typical result, the administration of 1 nmol (1 μg) of vasopressin in solution was followed within a few minutes by an antidiuresis, which lasted about 20 to 30 minutes (Fig. 3). After the rate of urine flow had returned to starting levels, one-fifth the dose (0.2 nmol) of vasopressin was given orally in an azoaromatic polymer-coated capsule. Antidiuresis occurred only after a delay of 100 minutes and lasted 1 hour. In 17 rats, the mean time to peak antidiuresis after the oral administration of vasopressin in solution was 23.5 ± 3.5 (SE) minutes, while after vasopressin protected by the azoaromatic polymer the mean time to maximum antidiuresis in 19 rats was 170 ± 15.5 minutes. The difference is highly significant ($P < 0.001$) by Student's t test. These data indicate that the transit time of the capsule averaged about 2.5 hours. There appeared to be a bimodal distribution of the time of delivery of the vasopressin, with a cluster of times around 2 hours and others at longer intervals ranging from 5 to 8 hours. The variable time of the vasopressin response makes it difficult to combine the results on individual animals for comparison with the controls.

Smaller pellets containing 1 IU of insulin were prepared by evaporating 10 μl of a 100 U/ml solution of insulin (Eli Lilly) onto a strip of Whatman #3 filter paper (1 × 3 mm). After the strip dried, it was coated by dipping into a 15% solution of azoaromatic polymer. The pellets were kept in the refrigerator overnight before use. At 5 p.m. 1 ml of water or mineral oil as lubricant, followed immediately by a control or azoaromatic polymer-protected insulin pellet, were given by a 16-gauge gavage needle to nonanesthetized rats allowed food and water ad libitum. There was no significant difference in the apparent transit times between the two lubricants. The rats were left undisturbed in their cages except for hourly blood samples from the tail. Figure 4 illustrates a typical experiment in which oral insulin caused a sustained fall in the blood levels of glucose in two diabetic rats. There was no decrease in blood glucose over the same time period in diabetic rats given control pellets.

The action of orally administered insulin in normal (nondiabetic) rats was less marked. In six normal rats given control pellets without insulin or insulin pellets not coated with azoaromatic polymer, the lowest blood glucose levels in the next 9 hours varied from 83 to 99% [mean 90.6 ± 2.3 (SE)] of the initial value (Fig. 5). After oral administration of azoaromatic polymer-

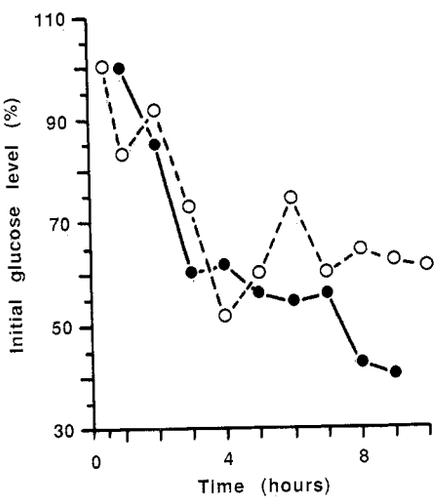

Fig. 4. The effect on blood glucose of the oral administration of an azoaromatic polymer-coated pellet containing 1 IU (about 28 nmol/kg body weight) of insulin on the blood glucose levels in two rats made diabetic with streptozotocin. The initial blood glucose levels were 400 mg per 100 ml (○---○) and 290 mg per 100 ml (●—●), respectively. The falls in blood glucose became significant (decrease >15%) 3 hours after the administration of the insulin. The pellets were administered at 5:15 p.m. and one-drop samples of tail blood were collected directly on Dextrostix Reagent Strips (Ames Division, Miles Laboratories) at 0.5 and 1 hour later and at hourly intervals thereafter for a total of 9 hours. The blood glucose was estimated on the Dextrostix with a Glucometer. The coefficient of variation of duplicate determinations was 8%. Agreement with results in a Beckman Glucose Analyzer was within 10% at all values of glucose tested (30 mg/dl to 390 mg/dl) (*21*).

coated pellets of insulin at 5 p.m. to a series of 12 normal rats, the lowest blood glucose values ranged from 64 to 93.5% (mean 79.6 ± 2.4) of the initial values. Normal rats given 0.1 and 1 IU of insulin intraperitoneally had mean low values of blood glucose of 61.2 ± 5.3 and 36.8 ± 3.1, respectively. The differences between the means in Fig. 5 are significant at $P < 0.02$.

The oral administration to rats of vasopressin and insulin protected with azoaromatic polymer coatings produced biological responses, antidiuresis, and hypoglycemia, respectively, characteristic of the peptide hormones. These results show that the azoaromatic polymer delivery system operates in principle. However, the responses occurred at variable times, from 1 to 9 hours after the administration of the protected peptide; therefore, results with different animals cannot be combined. There is no explanation at present for the variation in time of delivery of the peptides. In some animals that did not respond for 10 hours or more, the pellets were found in the stomach debris upon autopsy. Thus, holdup in the stomach may explain some of the variation. From direct observation of transit time of a dye solution in water-loaded rats, the minimal transit time of liquids to the ileo-cecal junction is about 1 hour. However, solids, such as polystyrene microspheres, have been shown to reach the end of the small intestine with a transit time of 195 minutes (*17*). Until a combination of an animal model and an azoaromatic polymer with a reproducible delivery time is found, bioavailability studies will be difficult to carry out because the peak of absorption of the drug can be missed.

Many characteristics of this new delivery system remain to be established. These include the relation between the chemical composition and the physical properties of the azoaromatic polymers and their ability to deliver drugs to the colon, the effect of preparation of the azoaromatic polymer-coated pellets on the activity of the drugs, the ability of the azoaromatic polymers to protect peptide drugs against the action of digestive enzymes, the rate of release of drugs after degradation of the polymer, the influence of the thickness and the geometry of the azoaromatic polymer coating on the release of the drug, the action of intestinal microflora on the drugs themselves, and the passage of the drugs from the site of release in the intestine into the blood.

Oral administration of drugs in azoaromatic polymeric coating can, in theory, be used for the delivery of a variety of new peptide drugs, provided that these drugs survive in the environment of the colon and are absorbed into the blood. These new drugs include analgesic peptides (endorphins and their analogs), contraceptive peptides (gonadotropin-releasing hormone and its analogs), growth-promoting peptides (growth hormone–releasing hormone and its analogs), oral vaccines, plasminogen-activating peptides, and others. Heparin and other digestible polysaccharide compounds could perhaps be given in this way as well. Codelivery of drugs with absorption adjuvants, such as 5-methoxysalicylate (*18*), may enhance the absorption of the drugs to the point of cost-effectiveness. Drugs in time-release forms may be coated with the azoaromatic polymer to combine oral administration with long duration of action. Azoaromatic polymers might also be used to deliver drugs to the colon for local action. These include anti-inflammatory agents, antibiotics, and chemotherapeutic compounds, some of which would be destroyed or almost completely absorbed in the small intestine and fail to reach the colon, while others may be too toxic for delivery by a systemic route.

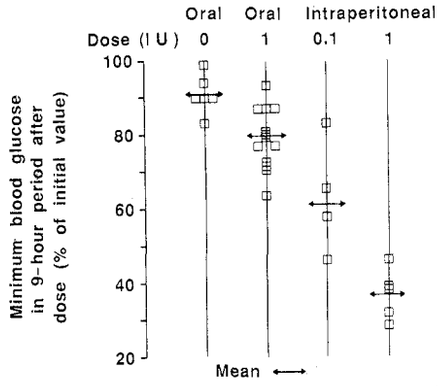

Fig. 5. Effect of control pellets, the oral administration of azoaromatic polymer-coated pellets containing 1 IU of insulin, and the intraperitoneal injection of 0.1 and 1 IU of insulin on the fall in blood glucose in normal rats. Control pellets were either coated pellets without insulin or insulin pellets without azoaromatic polymer coating. The doses were administered at 5 p.m. The room was darkened at 5:15. The initial blood sample was taken at 5:30. This value was taken as 100%. Subsequent blood samples were taken at 6 p.m. and at hourly intervals thereafter. The rats were allowed food and water ad libitum. Each point represents the lowest level of blood glucose, expressed as percent of the initial value, observed in each rat during the 9-hour period after the administration of the dose. The mean of each group of results is indicated by ⟷. The groups differ significantly from each other ($P < 0.02$; Student's t test).

The large intestine is drained by hepatic portal veins and by lymphatics. The oral delivery of insulin to the upper colon therefore resembles somewhat the natural route of delivery of pancreatic insulin, which is through the portal circulation to the liver. However, if a peptide drug is almost completely destroyed by the first pass through the liver, it would be advantageous to design an azoaromatic polymer that can deliver its load of drug in the lower colon, where most of the drainage is lymphatic (*19*). This may be possible by adjusting the thickness or composition of the coating or both.

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12. Human feces were used as a source of microorganisms to minimize the contact of the microflora with oxygen. Rat feces are hard pellets and are difficult to suspend quickly in the medium. Human feces can be suspended quickly in the medium under anaerobic conditions in a very short time. The literature (for example, *9*) shows that both rat and human microflora are able to reduce the aromatic azo bond with equal ease. The long incubation time of 3 to 8 days was necessary because the in vitro tests on films and threads of polymer were carried out with a relatively dilute suspension of microflora, in contrast to the very concentrated population in the colon, where reduction can occur within minutes. Direct observation of the reduction of an azoaromatic polymer was made by administering a solution of a water-soluble polymer, Poly Black (Aldrich Chemical), into the stomachs of a group of rats under the conditions of the vasopressin assay (*15*). The animals were killed at approximately hourly intervals. The front of the dye solution reached the cecum in about 60 to 75 minutes. Bleaching of the dye, indicating reduction, occurred about 5 mm upstream from the ileo-cecal junction. Although the dye continued to be delivered for up to 3 hours after administration into the stomach, the color never passed the zone of reduction at the ileo-cecal junction. This experiment indicated that the transit time of a liquid from the rat stomach to the ileo-cecal junction was about 60 to 75 minutes. Solid particles are known to require longer transit times (*17*). Recently Lundin (*20*) observed that after the direct instillation of DDAVP into various parts of the rabbit intestinal tract, the most absorption into the blood followed introduction of the peptide at the ileo-cecal junction.
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6 December 1985; accepted 14 July 1986

Direct Polyclonal Activation of Human B Lymphocytes by the Acquired Immune Deficiency Syndrome Virus

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When B lymphocytes from normal human peripheral blood were incubated for 1 hour with the retrovirus that causes the acquired immune deficiency syndrome (AIDS), the B cells showed marked proliferation and differentiation. Proliferative responses to the virus peaked on day 4 and appeared to be independent of accessory cells. This finding was repeated with three separate viral isolates, one of which was from a patient from Zaire. The magnitude of the observed responses was comparable to that seen with standard polyclonal B-cell activators. This phenomenon may be at least partially responsible for the polyclonal B-cell activation seen in patients with AIDS.

THE ETIOLOGIC AGENT OF THE ACquired immune deficiency syndrome (AIDS), known as human T-lymphotropic virus type III or lymphadenopathy-associated virus (HTLV-III/LAV), selectively infects and destroys the helper/inducer subpopulation of human T lymphocytes (*1*). This results in a severe crippling of the immune system at the T-cell level. There is good evidence that the T4 molecule is the receptor for the virus, not only on helper/inducer T cells but on a variety of cells and cell lines (*2*). Among the immunologic abnormalities that are thought to be a direct result of infection with the AIDS virus are lymphopenia, which is predominantly due to the quantitative decrease in helper/inducer T lymphocytes, decreased immunologic responses to soluble antigens, and decreased virus-specific cytotoxicity (*3*). In contrast to these depressive effects on the function of T cells, infection with the AIDS virus results in an intense polyclonal activation of B cells. This is evidenced by elevated serum levels of immunoglobulins G and A (IgG and IgA), the presence of circulating immune complexes, and an increased number of peripheral blood lymphocytes spontaneously secreting immunoglobulin (*4*).

Several mechanisms have been proposed to explain the polyclonal B-cell activation seen in patients with AIDS. Among them are the T-cell independent activation and transformation of B lymphocytes by Epstein-Barr virus (EBV) or cytomegalovirus in the absence of the normal immunoregulatory T-cell influences, the production of B-cell activating factors by AIDS virus–infected T cells, and a direct activation of B cells by the AIDS virus. An understanding of the interaction of the AIDS virus with B lymphocytes might provide additional insights into the immunopathogenesis of AIDS and, in particular, might suggest that receptor molecules other than T4 could play a role. In the present study we demonstrate that the AIDS virus can induce B-cell proliferation and immunoglobulin secretion to a degree that is comparable with any of the currently used polyclonal B-cell activators.

For these experiments, human peripheral blood mononuclear cells were obtained from healthy individuals who were seronegative for antibodies to the AIDS virus. They were greatly enriched for B cells by means of Hypaque-Ficoll separation and depletion of monocytes by passage through G-10 columns. Depletion of T cells was then achieved by rosetting with sheep red blood cells that had been treated with 2-aminoethylisothiouronium bromide (*5*). The resulting cell suspensions contained 70 to 85% B cells and less than 5% T cells, as determined by phenotypic and functional analysis with the use of monoclonal antibodies to the CD3 complex; a polyvalent antiserum to surface immunoglobulin; and proliferative responses to phytohemagglutinin (PHA), a pure T-cell mitogen, and whole, Formalin-treated *Staphylococcus aureus* Cowan strain I, a pure B-cell mitogen. While cell separation techniques such as sorting by FACS (fluorescence-activated cell sorting) are capable of providing more highly purified populations of B cells, the large numbers of cells required in our studies made such methods infeasible.

Supernatants containing different isolates of the AIDS virus were obtained by growing the viruses either in peripheral blood mononuclear cells that had been stimulated for 3 days with PHA or in the A3.01 cell line (*6*).

Samples of B cells were incubated for 1 hour with A3.01 supernatant containing AIDS virus strain FB-3, and B-cell proliferation, determined by [^{3}H]thymidine incorporation, was measured daily. As shown in Fig. 1, substantial B-cell proliferation was induced by the FB-3 containing supernatant. This proliferation was noted as early as day 2 and peaked on day 4 in all experiments. In contrast to the B-cell activation and transformation that have been described for EBV (*7*), the B-cell proliferation induced by FB-3 was a transient event and was no longer detectable by day 9 of culture. The magnitude of the response (9,000 to 28,000 cpm) was as great as that seen with any of the known T-cell independent human B-cell mitogens, such as *Staphylococcus aureus* Cowan strain I or EBV (*8*).

We next determined the concentration of virus that would result in the greatest prolif-

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