tern of deoxyribonuclease 1 cleavage sites along the whole length of the 200 base pairs].

The DNA lost from the ends of chromatin fragments during digestion of nuclei with micrococcal nuclease appears in acid-soluble form. On prolonged digestion a plateau is reached, similar to that reported for digestion of sheared chromatin (*5, 13*) in which a limit of about 50 percent of the DNA becomes acid soluble. A study of the limit digest of sheared chromatin has led Van Holde *et al.* (*14*) to a model of chromatin structure in which 100-Å units containing 110 to 120 base pairs of DNA [the size of DNA in chromatin fragments isolated (*15*) from the limit digest] alternate with 240-Å spacer regions. This differs fundamentally from the model mentioned above (*2*) in which the 100-Å units contain 200 base pairs of DNA and are joined directly to one another. Our study of prolonged nuclease digestion (*16*) casts doubt on the significance of the limit at 50 percent of acid-soluble DNA, and thus calls into question any model based on the properties of the limit digest. Convincing, direct evidence of a 100-Å unit containing 200 base pairs of DNA has recently been obtained by Griffith (*17*).

Although chromatin prepared by the nuclease method suffers occasional single-strand breaks and some loss of DNA from the ends, it contains the same repeating structure as chromatin in the nucleus whereas chromatin prepared by shearing does not. Of course we have tried only one of the commonly used methods of shearing, and it may be argued that other methods are less harsh, but we suspect that any shearing sufficient to break covalent bonds in the DNA will have a deleterious effect. Some workers have preferred nuclease digestion to shearing in the preparation of chromatin (*18*) but most studies have been done on sheared material and it seems unavoidable that many of these studies will need to be repeated.

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23 December 1974

Insulin-Induced Augmentation of Lymphocyte-Mediated Cytotoxicity

Abstract. *Physiologic concentrations of insulin enhance the ability of cytotoxic lymphocytes to injure target cells. The effect of insulin closely resembles the action of cholinomimetics and guanosine 3′,5′-monophosphate upon this system. Since both insulin and cholinomimetics elevate intracellular concentrations of guanosine 3′,5′-monophosphate, a common mode of action is suggested.*

After sensitization with an allograft, the mouse develops thymus-derived (T) lymphocytes that are selectively cytotoxic in vitro for cells bearing donor alloantigens (*1*). By means of a similar in vitro system, rats immunized by allografts have been demonstrated to develop cytotoxic lymphocytes that appear to be T lymphocytes (*2*) since the cytotoxic cells do not adhere to glass bead columns (*3*) nor do they bear easily detectable surface immunoglobulin (*4*). Intimate contact between viable sensitized lymphocytes and target cells is a prerequisite for cytotoxicity [lymphocyte-mediated cytotoxicity (LMC)]. Increases in the concentration of adenosine 3′,5′-monophosphate (cyclic AMP) inhibit LMC (*2, 4, 5*), whereas cyclic AMP depletion (*4*), exogenous 8-bromoguanosine 3′,5′-monophosphate (*4*), or cholinergic agonists (*2, 4, 6*) that elevate levels of guanosine 3′,5′-monophosphate (cyclic GMP) in lymphocytes enhance cytotoxicity via an effect upon the attacking and not the target cells (*4, 6*). Recent evidence indicates that mononuclear leukocytes bear specific receptors for insulin (*7–9*). Furthermore, stimulation of tissues (*10, 11*) with insulin has been reported to result in elevated intracellular levels of cyclic GMP. Since previous studies indicated that enhancement of LMC should result from elevated levels of cyclic GMP in effector cells (*4*), the effect of physiologic concentrations of insulin upon LMC was studied. The augmented LMC reported here, resulting from an action of insulin upon the effector cell, supports and extends the implications of previous studies which indicated a crucial role for cyclic nucleotides in regulating LMC (*2, 4, 6*).

The agents listed were obtained from manufacturers: bovine crystalline insulin and atropine sulfate (Sigma Chemical, St. Louis); RPMI-1640 medium and fetal calf serum (Grand Island Biological, Grand Island, New York); fluorescein isothiocyanate and unabsorbed guinea pig complement (Nutritional Biochemical, Cleveland); deoxyribonuclease (Pentex Biochemical, Kankakee, Illinois); and ^{51}Cr-labeled sodium chromate (Nuclear Chicago, Chicago). Streptozotocin was a gift from P. Schein, National Institutes of Health, Bethesda, Maryland.

The cytotoxic action of alloimmune splenic lymphocytes on thymocytes bearing alloantigens to which they are sensitized was determined by a previously described modification (*2, 4*) of the technique of Brunner *et al.* (*12*). In brief, splenic lymphocytes were har-

vested from Lewis (L) rats 7 days after they received skin grafts from (Lewis × Brown Norway)F_1 rats (LBN rats). The sensitized splenocytes were used as attacking cells, and ^{51}Cr-labeled Brown Norway thymocytes served as target cells. Cytotoxicity was quantitated as percentage of specific lysis as determined by ^{51}Cr release from attacking and target cell mixtures after 4 hours of interaction of 5×10^6 attacking cells with 5×10^4 target cells. Culture media contained 10 percent (by volume) heat-inactivated serum obtained either from severely insulin-deficient, diabetic, streptozotocin-treated rats (*13*) or from lots of fetal calf serum that had insulin concentrations of < 1.5 microunit/ml as determined by the double antibody technique (*14*).

Insulin diluted in medium just before use was interacted with the sensitized lymphocytes for varying times at room temperature before introduction of the target cells. Insulin did not injure either target or attacking cells as determined in ^{51}Cr release studies.

Three pooled sensitized L rat spleens were incubated with heat-inactivated rabbit antiserum against rat immunoglobulin and complement and then treated with deoxyribonuclease as described (*4*). A 41 percent reduction of the total cell population and total elimination of immunoglobulin-bearing splenocytes, as determined by inspection of suspensions incubated with fluorescein isothiocyanate-conjugated rabbit antiserum against rat immunoglobulin G (*15*), were achieved by this treatment.

Physiologic concentrations of insulin augmented LMC (Table 1). Enhancement of LMC was obtained when the concentration of the agent was confined to the physiologic range of 10^{-8} to $10^{-11}M$ (Table 1). The insulin concentrations in the fetal calf serums used were at the lower limits of detection by radioimmunoassay (0.75 to 1.5 microunit/ml or $10^{-11}M$); therefore, the final concentration present in the culture media was $10^{-12}M$ before addition of exogenous insulin. Insulin denatured by boiling for 12 hours did not alter cytotoxicity, which indicates that stimulation by insulin is dependent upon the intact configurational properties of this ligand. Previous studies indicated that stimulation of the muscarinic cholinergic receptors of attacking cells also augments LMC (*2, 4, 6*); however, the muscarinic antagonist atropine did not abrogate insulin-induced augmentation of LMC. Thus, it would appear that the effect of insulin does not derive from stimulation of the lymphocyte muscarinic receptor. Physiologic concentrations of insulin did not induce nonsensitized lymphocytes to injure target cells (Table 1). Deletion of immunoglobulin-coated lymphocytes (B lymphocytes), however, did not prevent insulin from enhancing LMC (Table 1). Furthermore, deletion of B lymphocytes did inhibit cytotoxicity in untreated mixtures, an observation consistent with our earlier data (*4*).

Table 1. Effect of insulin concentration on augmentation of lymphocyte-mediated cytotoxicity (LMC). In the first two experiments, insulin-deficient serum harvested from streptozotocin-treated rats was the protein source in the culture medium; fetal calf serum was the protein support in the last two experiments; Ig+, immunoglobulin-positive.

Specific lysis without added insulin (%)	Insulin (M)	Insulin-induced LMC increase (%)
	L cells after LBN → L skin graft	
	$\geq 10^{-7}$	≤ 5
	10^{-8}	32 ± 2
44 ± 3	10^{-9}	73 ± 4
	10^{-10}	52 ± 4
	10^{-11}	47 ± 2
	$\leq 10^{-12}$	≤ 5
	L cells after LBN → L skin graft	
	$\geq 10^{-7}$	≤ 5
	10^{-8}	29 ± 2
41 ± 1	10^{-9}	44 ± 0
	10^{-10}	77 ± 4
	10^{-11}	58 ± 2
	$\leq 10^{-12}$	≤ 10
	L cells after LBN → L skin graft depleted of Ig+ cells	
	10^{-9}	42 ± 4
39 ± 3	10^{-10}	52 ± 3
	10^{-11}	38 ± 2
	Unsensitized L cells	
	10^{-9}	0
0 ± 1	10^{-10}	0
	10^{-11}	0

In Table 2, the augmentation of LMC produced by insulin is seen to be completely dependent upon a short period of prior incubation of the attacking cells with this agent. Augmentation was not observed if the attacking cells were incubated with insulin for more than 7 minutes before introduction of the target, nor if insulin was added after both attacking and target cells were mixed. These data strongly suggest that insulin augments LMC via an effect upon the attacking cells alone, since the addition of insulin to mixtures of attacking and target cells is ineffective. Similarly, the precise timing of the insulin effect also makes it unlikely that this action of insulin is a nonspecific one resulting from merely an improvement of cellular metabolism.

The time-dependent and optimal dose-dependent enhancement of LMC mediated by insulin closely resembles the effect of cholinergic (*4, 6*) agonists and cyclic GMP (*4*) upon this system. The similarities noted in cholinergic- and insulin-induced augmentation of LMC are probably substantive and based upon parallel alterations of cyclic nucleotide metabolism in lymphocytes. Previous studies have indicated that the levels of cyclic nucleotides within the attacking cells at the moment of interaction with the target cells modulate LMC (*2, 4*). Since both insulin (*10, 11*) and cholinergic agonists (*11, 16*) transiently elevate cyclic GMP levels in tissues it is likely that increased concentrations of intracellular cyclic GMP, acting as a classical second messenger for insulin and cholinomimetics, mediate the augmented cytotoxicity produced by these agonists.

The distribution of insulin receptors among mononuclear leukocyte subpopulations is incompletely defined. Recent studies have indicated that peripheral blood lymphocytes prepared by passage

Table 2. Dependence of insulin augmentation of LMC on prior exposure of attacking cells to insulin. Data are percentages of insulin-induced LMC increase; L cells harvested after LBN → L skin grafts were used in both experiments. Exposure times of zero minutes or less indicate that insulin was added concurrently with target cells or afterward. Data for two experiments are shown.

Insulin (M)	Insulin-induced augmentation of LMC (%) after exposure of attacking cells to insulin for									
	8 min	6 min	5 min	4 min	3 min	2 min	1 min	0 min	−1 min	−2 min
10^{-9}	0	16 ± 3	20 ± 3	42 ± 0	73 ± 4	36 ± 3	42 ± 0	0	0	0
10^{-10}	0	15 ± 2	20 ± 2	47 ± 1	52 ± 4	47 ± 2	42 ± 2	0	0	0
10^{-11}	0	19 ± 4	16 ± 1	47 ± 3	47 ± 2	52 ± 4	36 ± 1	0	0	0
10^{-9}	0	0	14 ± 1	26 ± 3	33 ± 2	44 ± 0	57 ± 1	0	0	0
10^{-10}	0	0	12 ± 2	19 ± 1	37 ± 0	77 ± 4	43 ± 2	0	0	0
10^{-11}	0	0	9 ± 2	32 ± 4	44 ± 2	58 ± 2	24 ± 1	0	0	0

through glass bead columns (*7*) or by Ficoll Hypaque gradient separation (*8, 11*) bear insulin receptors. In contrast, human peripheral blood lymphocytes (*17*) or rat spleen lymphocytes (*11*) prepared by passage through a nylon wool column do not bear insulin receptors; however, receptors emerge upon the nonadherent effluent cells during lymphocyte transformation induced by concanavalin A (*18*). Since the primary consequence of passing mononuclear cells through nylon wool columns is deletion of B lymphocytes (*19*) and macrophages (*20*), integration of these data would indicate that cells adherent to nylon wool bear insulin receptors (*9*) whereas nonstimulated splenic and peripheral blood T lymphocytes lack insulin receptors unless the nylon wool column damages insulin receptors. The present data indicate that immunoglobulin-negative cytotoxic lymphocytes (almost certainly T lymphocytes) bear functional insulin receptors. The mononuclear cells of patients with adult onset diabetes (*21*) exhibit deficient insulin receptors. Since insulin profoundly augments LMC, the propensity of diabetic patients to suffer from recurrent and severe microbial infection may be due in part to altered lymphocyte metabolism resulting in suboptimal lymphocyte responsiveness.

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23 August 1974

Permanent Translocation Heterozygosity and Sex Determination in East African Mistletoes

Abstract. Viscum fischeri *has* 2n = *23 chromosomes in male plants. These form 7 bivalents and a translocation chain of 9 chromosomes during meiosis. Pollen with 11- and 12-chromosome genomes is thus produced. Female plants have* 2n = *22 chromosomes and produce 11 bivalents during meiosis. Sex determination is technically a rare multiple X–multiple Y type, but more importantly it provides the mechanism whereby permanent translocation heterozygosity is maintained in the system. In a second species,* Viscum engleri, *male plants have* 2n = *28 chromosomes, associating as 11 bivalents and a ring of 6 chromosomes at meiosis.*

Recently we described an unusual case of translocation heterozygosity in an East African mistletoe, *Viscum fischeri* Engl. (*1*). A chromosome number of $2n = 23$ was determined in male plants undergoing meiosis. In first meiotic metaphase a configuration of 7 bivalents and an open, multivalent chain of 9 chromosomes was consistently observed (Fig. 1). A regular, alternate (zigzag) orientation of the chain resulted in 4 : 5 disjunction at first anaphase, so that 11- and 12-chromosome genomes were transmitted via the pollen. The 11-chromosome set characteristically was comprised of 10 metacentrics or submetacentrics and 1 acrocentric, while the 12-chromosome set had 9 metacentrics or submetacentrics and 3 acrocentrics [for additional photographic documentation, see (*1*)]. The exceptionally large chromosomes in *Viscum* greatly facilitate analysis (*1*).

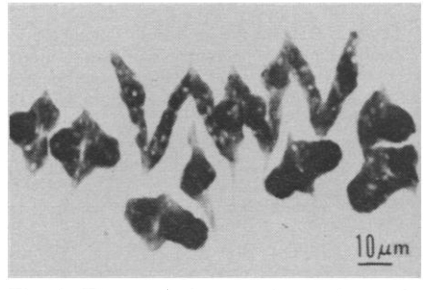

Fig. 1. First meiotic metaphase of a male plant of *V. fischeri* illustrating the chain of 9 chromosomes and the 7 bivalents. The chain is oriented in typical zigzag fashion prior to 4 : 5 disjunction at first anaphase; chiamata are visible as the rounded enlargements along the chain.

We also pointed out that if the translocation heterozygosity in *V. fischeri* is permanent, then the system differs significantly from other permanent translocation heterozygotes such as species of *Oenothera* and *Isotoma* (*2*). In *V. fischeri* the system involves structurally and numerically different genomes in which some chromosomes are acrocentric, and an open (chain) multivalent, whereas in other translocation heterozygotes even-numbered ring-forming genomes with metacentric chromosomes are the rule. It was also noted that *V. fischeri*, being dioecious, is an obligate outcrosser, whereas inbreeding is characteristic of other permanent translocation heterozygotes.

Since the species is dioecious we also noted that the numerically different genomes were possibly related to the sex-determining system. One essential aspect for further study, therefore, was the karyological structure and meiotic behavior of female plants, which could not be determined from the original materials. A study of mitotic cells from shoot apices in female plants from two geographically diverse populations in