

Persistent Cutaneous Insulin Allergy Resulting From High-Molecular-Weight Insulin Aggregates

ROBERT E. RATNER, TERENCE M. PHILLIPS, AND MALCOLM STEINER

Cutaneous insulin allergy remains a clinical problem despite the use of highly purified human insulins. We used in vitro lymphocyte-transformation studies to examine the reactivity of various insulin formulations in diabetic patients with ($n = 4$) and without ($n = 8$) cutaneous allergies. Nonspecific response to concanavalin A demonstrated a >40-fold response in both groups. Control patients did not respond to the addition of commercial insulin preparations (stimulation index [SI] <4), whereas allergic patients had an 11-fold response to beef ($P < 0.01$), a 10-fold response to pork ($P < 0.01$), and a 6-fold response to human ($P < 0.01$) insulins. This response was limited to a single insulin manufacturer's preparations and was uniform in all three species tested. Efforts to identify the offending agent revealed no lymphoblast transformation when crystalline insulin was used or when commercial preparations were purified to a single peak by high-performance liquid chromatography (HPLC). Pure crystalline insulin dimers of beef, pork, and human species were tested; control subjects responded with mean SIs of 1.9, 1.9, and 1.8, respectively, whereas allergic patients showed greater reactivity to beef (SI 7.3) and pork (SI 14.8). The lymphoblast-transformation response to crystalline human dimer was dose dependent with mean SIs of 0.9 at low concentration (2.8 ng/ml) and 19.2 at a higher concentration (20.4 ng/ml). The commercial insulin preparations were run on size-exclusion HPLC to determine high-molecular-weight aggregate content. Independent of species, a single manufacturer had products demonstrating aggregate levels 3- to 6-fold higher than those found in other manufacturers' preparations. Our in vitro data suggest that insulin aggregates rather than native monomers cause

cutaneous allergy and that antigenicity of commercial preparations correlates with the relative concentration of aggregates. In vivo testing is needed to confirm our results. *Diabetes* 39:728–33, 1990

Clinically significant cutaneous insulin allergy is found despite the use of highly purified human insulin preparations. In the past, use of mixed beef-pork products resulted in a 40–60% incidence of local reactions (1,2). The allergic response was transient in most cases, and all but 1–2% remained localized (3). Frequently, such patients show a history of atopy, with drug or food allergies in as many as 50% of patients (4). Because these allergies were generally self-limited and mild, they were clinically significant in only 3–5% of patients (2,4).

Identification of culpable commercial insulin components has been achieved with in vivo methods, i.e., patch testing (5) and in vitro testing with lymphocyte-transformation studies (4,6). Offending agents in these preparations have included biological contaminants, i.e., proinsulin (4), and additives, i.e., zinc (7) or protamine (8). As a rule, local insulin allergies have been effectively treated by switching therapy to highly purified species-specific insulin preparations (4,6).

We studied a series of patients in whom evidence of cutaneous allergy persisted despite use of highly purified commercially available human insulins. In vitro lymphocyte transformation studies were performed to identify the specific component of the commercial preparations responsible for induction of the allergic response.

RESEARCH DESIGN AND METHODS

Four patients with a history of localized urticarial insulin allergy were evaluated in the Diabetes Center of the George Washington University Medical Center. None of the patients gave a history of atopy or allergies to other drugs or food. The severity of the clinical response was sufficient for each individual to seek medical attention for that problem. Cutaneous insulin allergy was clinically confirmed by intradermal

From the Division of Endocrinology and the Immunochemistry Laboratory, Department of Medicine, George Washington University Medical Center, Washington, DC.

Address correspondence and reprint requests to Robert E. Ratner, MD, Division of Endocrinology, George Washington University Medical Center, 2150 Pennsylvania Avenue NW, Washington, DC 20037.

Received for publication 28 September 1987 and accepted in revised form 22 January 1990.

TABLE 1
Duration of diabetes and type of insulin therapy
in diabetic patients

Patients	Duration of diabetes (yr)	Insulin therapy
Allergic 1	16	Beef-pork, purified pork, human
2	3	Beef-pork, human, beef
3	1	Human
4	26	Beef-pork, purified pork, human
Mean	11.5	
Control 1	15	Beef-pork, purified pork
2	12	Beef-pork, human
3	5	Beef-pork
4	19	Beef-pork
5	14	Beef-pork, human
6	14	Beef-pork, human
7	23	Beef-pork, human
8	16	Beef-pork, human
Mean	14.75	

skin testing, which was performed by injection of 0.1 ml of a 1:100 dilution of beef, pork, and human insulins as the test allergens with the appearance of a wheal and flare response >15 mm diam. Eight diabetic patients with no previous history of insulin allergy but similar exposure to animal insulin preparations were used as control subjects for the *in vitro* studies (Table 1).

Commercial regular insulin preparations of purified beef, pork, and human origin representing the three manufacturers supplying insulin in the United States were tested in a lymphoblast-transformation system. In addition, pure crystalline insulin (lot 615-242-270-3) and beef (lot 615-JF9-86), pork (lot 615-IF9-41), and human (lot 615-3UO-99) insulin dimers (provided by R. Chance, Lilly, Indianapolis, IN) were subsequently used as stimuli.

Pork, beef, and synthetic human insulins were dialyzed in 1000-M, cutoff dialysis tubing (Fisher, Pittsburgh, PA) against 0.1 M sodium acetate, pH 7, for 5 h with three buffer changes. The insulins were then passed through a TSK 3000 size-exclusion column (Beckman, Fullerton, CA) that was connected to a Beckman 340 isocratic high-performance liquid chromatography (HPLC) system. The elution profile was monitored at 280 nm, and the profile was developed at 1 ml/min under controlled conditions.

Lymphocytes were separated from whole citrated blood by centrifugation on Ficoll-Hypaque density gradients. The lymphocytes were harvested, checked for viability by trypan blue exclusion, and counted. The cell suspension was adjusted to 1×10^6 cells/ml in Hanks' minimal salt medium, which had been supplemented with 10% heat-inactivated fetal calf serum. The lymphocyte-transformation assay was performed as a modification of the technique described by Schellekens and Eijssvoogoz (9). Briefly, 1-ml aliquots of the lymphocyte suspension were placed in plastic culture tubes and supplemented with 100 μ g of each of the various types of insulin preparations. The cells were mixed and incubated at 37°C for 72 h, washed twice in medium, and then pulsed for another 18 h with 0.2 μ Ci of [^{3} H]thymidine. After this incubation, the cells were washed three times in medium and harvested with an automated cell harvester (Skatron,

Sterling, VA). The incorporated radioactivity present in each cell pellet was measured in a Beckman LS3801 scintillation counter. All tests were performed in triplicate. An activation control, in which an aliquot of both patient and control cells was incubated with 5 μ g/ml concanavalin A, was performed with every assay to check the viability and reactivity of the cell cultures. Reactivity was expressed as a stimulation index (SI) or stimulated counts per minute divided by the background counts per minute for each cell culture system. The coefficient of variation was 3.5%.

Commercial insulin preparations were acidified to pH 3.5 with hydrochloric acid and chromatographed on a 9.4-mm $\times$ 25-cm Zorbax GF-250 column (Du Pont, Wilmington, DE) connected to a Beckman 340 isocratic HPLC system in an operating buffer of 69% 0.4 M ammonium bicarbonate and 31% acetonitrile. The flow rate was maintained at 0.8 ml/min, and the study was performed at room temperature. The detector was set at 280 nm with full-scale absorbance set at 0.08 U.

RESULTS

Cutaneous insulin allergy manifested itself in our four allergic patients at variable times in the course of therapy, ranging from the 1st wk of insulin therapy in patient 3 to 25 yr after initiation of therapy in patient 4. In general, allergic patients had been previously exposed to animal forms of insulin of various levels of purity from manufacturer A. However, patient 3 developed a cutaneous allergy despite limited exposure to manufacturer A's human insulin. Allergy was confirmed in this patient by excisional biopsy in which eosinophilic, mast cell, and histiocytic infiltration of the dermis and perivascular regions were noted together with marked edema.

Control patients had a similar duration of insulin-requiring diabetes (14.75 vs. 11.50 yr for allergic patients) and had a similar exposure history to animal preparations of insulin of varying purity but lacked clinical evidence of allergy.

In vitro lymphoblast-transformation responses to mitogenic stimulation with concanavalin A were demonstrated in all control and allergic patients, confirming the viability and responsiveness of the cell culture system (Fig. 1). Figure 2 illustrates the lymphoblast-transformation response of pa-

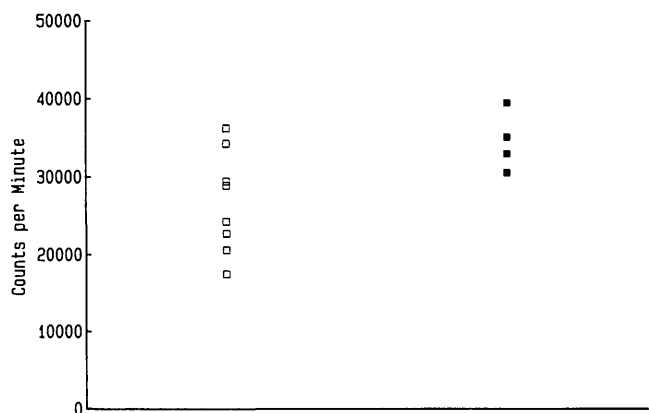


FIG. 1. Lymphoblast-transformation response to concanavalin A stimulation in control ($\square$; $n = 8$) and diabetic ($\blacksquare$; $n = 4$) lymphocyte cultures.

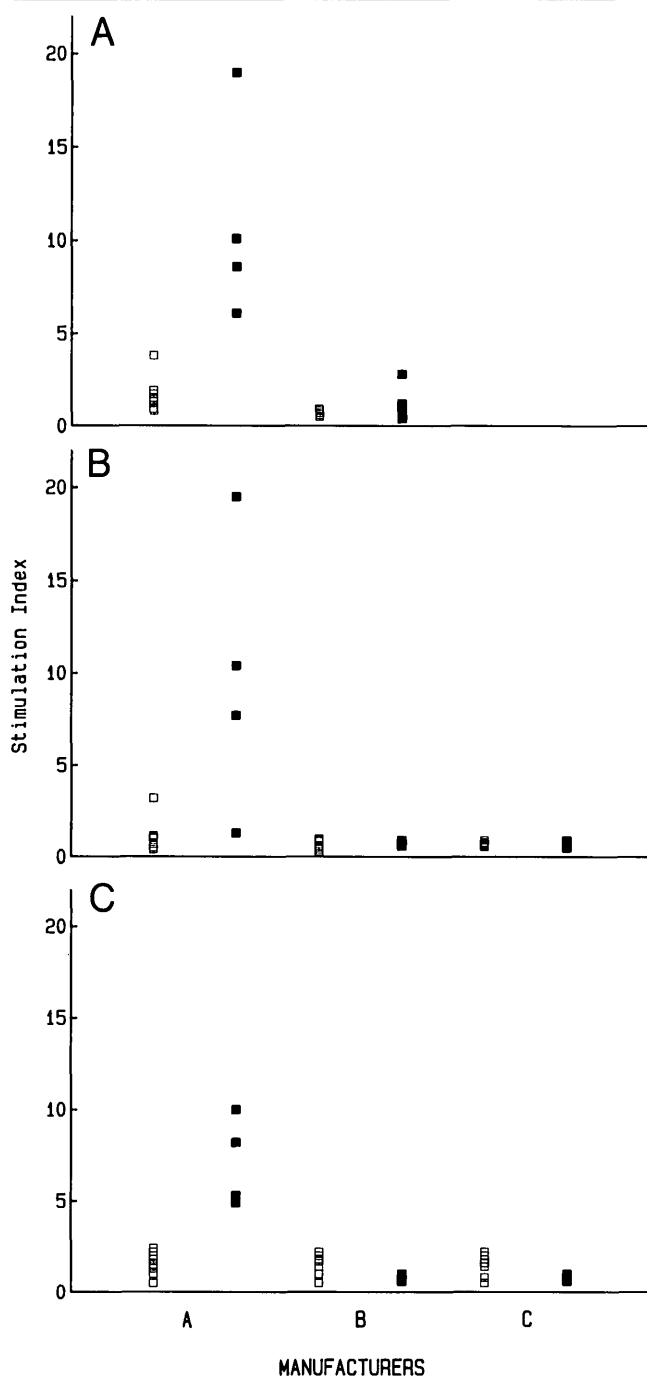


FIG. 2. Lymphoblast-transformation response to commercial insulin preparations separated by species (A, beef; B, pork; and C, human) and commercial manufacturer. □, Diabetic patients; ■, control subjects.

tients and control subjects to commercially available off-the-shelf preparations of beef, pork, and human insulins from three manufacturers. Control lymphocytes showed no lymphoblast transformation to any of the commercial preparations. In contrast, allergic patients' lymphocytes had a significantly greater lymphoblast transformation (beef, $P < 0.01$; pork, $P < 0.01$; and human, $P < 0.01$), determined by Wilcoxon rank-sum testing, when stimulated by commercial insulin preparations from manufacturer A, regardless of insulin species. Commercial preparations by manufac-

turers B and C generated no significant lymphoblast transformation in either control or patient lymphocytes.

In an effort to identify the specific component of the commercial preparations responsible for antigenic stimulation, pure crystalline human, low-zinc crystalline, and chromatographically purified insulin preparations were tested. Figure 3 reveals no lymphoblast response to any of the purified or crystalline insulins in either allergic or control samples.

Pure crystalline insulin dimers of beef, pork, and human origin were tested next to determine if polymeric forms of insulin might serve as antigenic stimuli. Figure 4 reveals no lymphoblast transformation in control lymphocytes, whereas patients' lymphocyte responses closely mirrored the response to commercial brand A. Beef dimer resulted in a mean SI of 7.3 in allergic patients compared with 1.95 in control. Pork dimer had a mean SI of 14.75 in allergic patients compared with 1.875 in control subjects ($P < 0.01$). Human insulin dimer showed a dose-dependent response with no lymphoblast transformation at a low concentration of 2.8 ng/ml, but it showed a significant response (SI 19.2 in allergic patients vs. 2.15 in control subjects, $P = 0.01$) when antigen concentration was increased to 20.4 ng/ml (Fig. 5).

These data suggest that the relative dimer composition of commercial insulin preparations may explain the variable response of insulins from one manufacturer to another. Size-exclusion HPLC of commercial insulin preparations was performed in an effort to determine whether dimer concentrations differed from one manufacturer to another. Figure 6 is an illustrative chromatogram of commercial pork and human insulins from manufacturer A compared with the elution profiles of crystalline human insulin monomer and dimer. Table 2 delineates dimer levels expressed as a percentage of total insulin obtained by this technique in commercial insulin preparations obtained from all three manufacturers. Products of manufacturer A, independent of insulin species, revealed dimer concentrations 3- to 6-fold higher than the corresponding insulin preparations from either manufacturer B or C. Concentration of insulin dimer exceeded the threshold for lymphoblast transformation only in those preparations from manufacturer A.

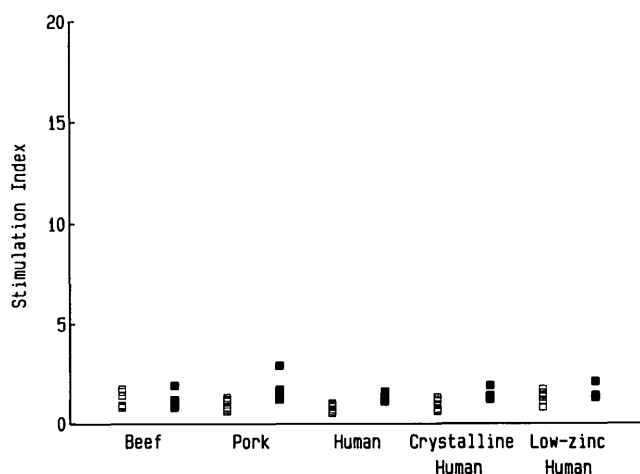


FIG. 3. Lymphoblast-transformation response to crystalline insulin and chromatographically purified insulins of different species in diabetic patients (■) and control subjects (□).

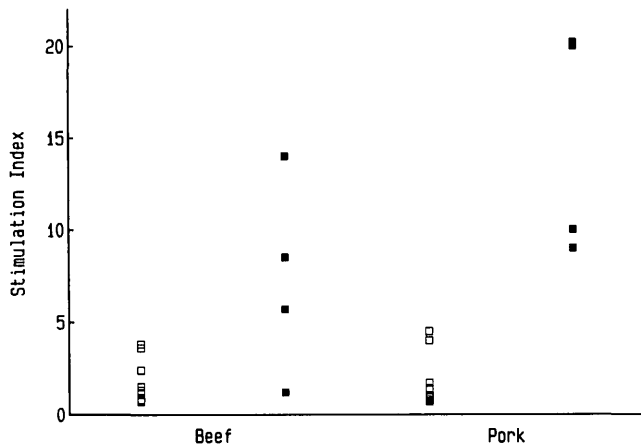


FIG. 4. Lymphoblast-transformation response to beef and pork crystalline insulin dimers in diabetic patients (■) and control subjects (□).

DISCUSSION

The introduction of insulin therapy was soon followed by the report of cutaneous insulin allergy (10). Subsequently, limited allergic response was seen in as many as 60% of treated patients (1). The frequent occurrence of allergy in the past could be partially explained by contaminants in older insulin preparations before the institution of repeated crystallization and purification techniques. The presence of gastrointestinal hormonal contaminants and proinsulin with subsequent antibody generation has been described (11,12). Although beef and pork insulin share extensive homology with human insulin, the C-peptide components of animal insulin differ markedly. Until the mid-1970s, with the introduction of monocomponent insulin preparations, proinsulin contamination in commercial preparations ranged from 3 to 6% (4).

The introduction of human insulin preparations resulted in decreased observation of clinical insulin allergy (13), but individual reports of its occurrence continue to be published (14). The persistence of this response would suggest that the allergy is related to either a contaminant, a preservative, or an altered form of the insulin molecule. Clinical allergies occurring in response to protamine (8) and zinc (7) have been described. High-molecular-weight insulin aggregates have been identified in the blood of insulin-treated diabetic patients, with therapeutic insulin preparations as their source (15,16). These high-molecular-weight forms of insulin have been found to generate circulating antibodies in insulin-treated individuals (17).

The allergic response results from either an IgE-mediated reaction, a cell-mediated delayed hypersensitivity, or an antibody-mediated Arthus reaction (18). In one study, diabetic patients with clinical allergies had significantly higher insulin-specific IgE levels compared with nondiabetic and nonallergic diabetic control subjects (5). However, 50% of allergic patients studied had IgE levels that fell within the normal range, suggesting a cell-mediated allergic response in these patients. Our four allergic patients were all IgE⁻ (data not shown) and lacked antibodies directed against high-molecular-weight insulin aggregates (kindly performed by D.C. Robbins, University of Vermont, Burlington). We conclude that their allergy is due solely to cell-mediated delayed

hypersensitivity. Our allergic patients had no lymphoblast response to pure crystalline insulin monomer; low-zinc crystalline human insulin; or chromatographically purified beef, pork, or human insulin (Fig. 3). They also failed to demonstrate lymphoblast transformation when their lymphocytes were stimulated with protamine, methylparaben, or zinc (data not shown), which are common additives to insulin preparations. Positive lymphoblast-transformation responses to commercial insulin preparations suggest that a modification of the insulin molecule per se resulted in the antigenic stimulus.

Insulin undergoes spontaneous self-aggregation into high-molecular-weight aggregates consisting primarily of hexamers (36,000 M_r) and dimers (12,000 M_r ; 19). Forces influencing this polymerization process include insulin concentration, pH, the presence of zinc, and the nature of the anions in the medium. Hexameric insulin predominates in the presence of zinc in a neutral solution and appears to result from a stepwise polymerization scheme from monomer to dimer to tetramer to its final hexameric shape. Attractive forces within the dimer occur at both nonpolar and hydrophobic residues. Initial van der Waals interactions maintain the dimer; however, subsequent covalent bonding occurs between an NH_2 -terminal amino group from one molecule to either a glutamine or an asparagine residue in the A chain of the second molecule (20). This structure takes on added importance, because many of the hydrophobic residues involved in dimerization play a critical role in insulin-receptor binding (21).

When covalently bound insulin dimers were added to the lymphocyte cultures of our patients with clinical insulin allergy, an allergic response mimicking that seen with commercial insulin preparations was found. The observation that the lymphoblast-transformation response differed among the commercial manufacturers rather than the species of insulin suggests a manufacturing or postmanufacturing alteration of the insulin molecule itself. When analyzed by size-exclusion HPLC, insulin preparations of manufacturer A demonstrated elevated dimer concentrations compared with those products from other manufacturers. It is unclear whether the dimerization process occurs before patient handling or sub-

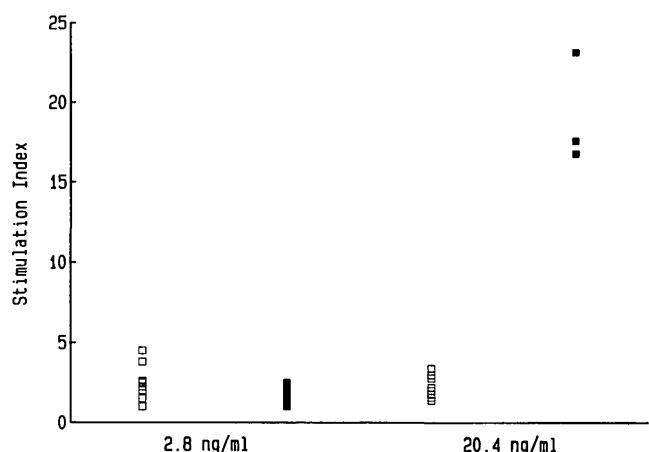


FIG. 5. Lymphoblast-transformation response to variable concentrations of human crystalline insulin dimer (2.8 vs. 20.4 ng/ml) in diabetic patients (■) and control subjects (□).

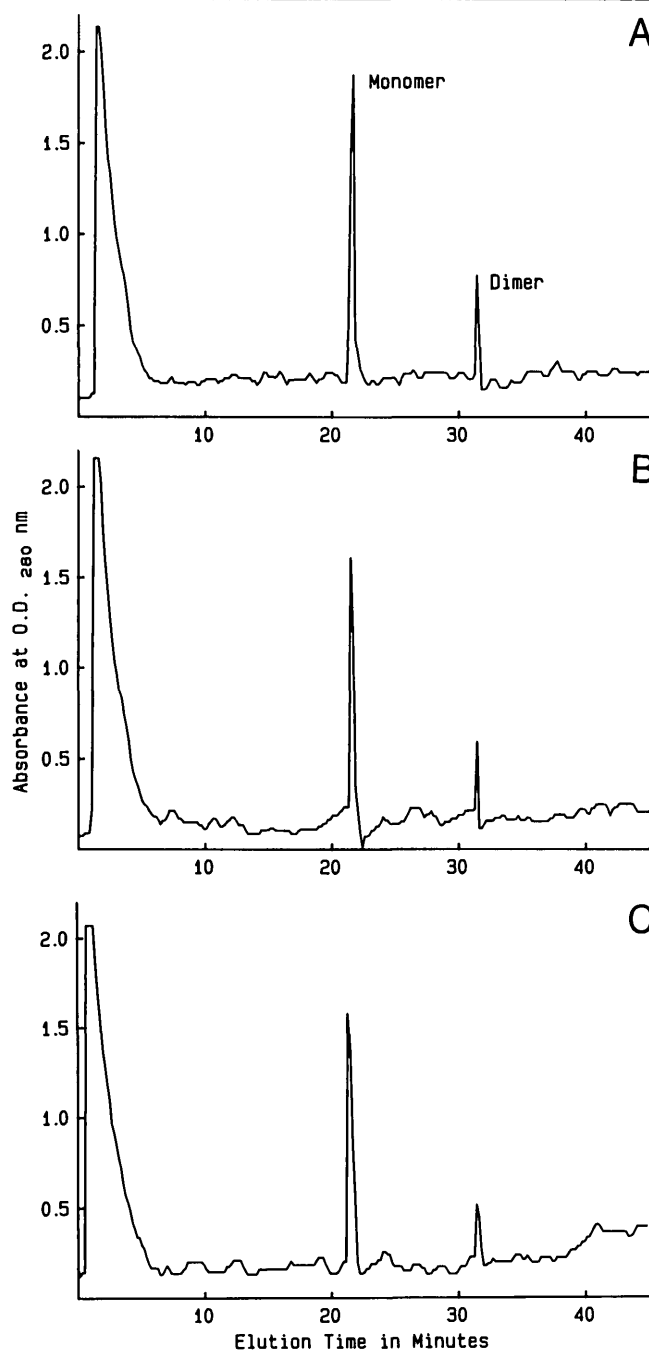


FIG. 6. Size-exclusion high-performance liquid chromatography of crystalline human insulin monomer and dimer standards (A) compared with chromatograms of commercial pork (B) and human (C) insulins from manufacturer A. O.D., optical density.

sequent to it. However, preparations from manufacturer A may lack the physical stability to prevent polymerization with subsequent insulin allergy or possible diminution of insulin effect.

Although clinical insulin allergy has become a less-frequent observation, new modalities of insulin delivery appear to magnify its propensity. In a small group of 20 diabetic patients treated with continuous subcutaneous insulin infusion, 25% developed new-onset insulin allergy that had not been present before pump therapy (22). Continued use of subcutaneous insulin-infusion pumps and newer investiga-

A

TABLE 2
Dimer levels in commercial insulin preparations

Insulin	Dimer level	
	ng/ml	%*
Manufacturer A		
Beef	7.2	1.7
Pork	9.6	2.3
Human	8.4	2.0
Manufacturer B		
Beef	2.4	0.6
Pork	2.1	0.5
Human	1.3	0.3
Manufacturer C		
Pork	2.5	0.5
Human	1.4	0.3
Crystalline dimer controls		
Beef	16.5	
Pork	23.1	
Human		
Low dose	2.8	
High dose	20.4	

*Dimer levels expressed as percentage of total insulin obtained by size-exclusion high-performance liquid chromatography.

B

C

tions with implantable systems raise the important issue of stability of insulin within a delivery system (23). Unique factors involved in the use of either implantable or external pumps include prolonged contact with plastic reservoirs and tubing, higher-temperature exposure, agitation as a result of a constant pumping motion, and higher insulin concentrations (24). The use of these novel insulin delivery systems will require the development of stable insulin preparations to avoid problems of polymerization with subsequent obstruction of the pump, precipitation of allergy, or diminution in biological activity of the insulin (25). Based on size-exclusion HPLC data, it appears that insulin preparations from either manufacturer B or C are superior because of the lower concentration of insulin dimer in the commercial preparations.

In conclusion, we identified a group of patients manifesting cutaneous insulin allergy resulting from a cell-mediated delayed hypersensitivity response to the dimeric form of insulin. Dimer formation varies from one manufacturer of insulin to another but not from one species of insulin preparation to another. The formation of high-molecular-weight insulin products may depend on either manufacturer or patient handling of the preparation. The development of physically stable insulin monomers may further decrease the clinical incidence of cutaneous insulin allergy and minimize complications associated with insulin-infusion therapy.

REFERENCES

1. Arkins JA, Engbring NH, Lennon E: The incidence of skin reactivity to insulin in diabetic patients. *J Allergy* 33:69-72, 1962
2. Lamkin N, Lieberman P, Hashimoto K, Morohashi M, Sullivan P: Allergic reactions to insulin. *J Allergy Clin Immunol* 58:213-23, 1976
3. Ross JR: Allergy to insulin. *Pediatr Clin North Am* 31:675-87, 1984
4. Kahn CR, Rosenthal AS: Immunologic reactions to insulin: insulin allergy, insulin resistance, and the autoimmune insulin syndrome. *Diabetes Care* 2:283-95, 1979
5. De Shazo RD, Mather P, Grant W, Carrington D, Frentz JM, Leug M, Lauritano AA, Falholt K: Evaluation of patients with local reactions to insulin with skin tests and in vitro techniques. *Diabetes Care* 10:330-36, 1987
6. White WB, De Martino SA, Yoshida T: Severe delayed inflammatory re-

- actions from injected insulin. *Am J Med* 74:909–14, 1983
7. Feinglos MN, Jegasothy BV: Insulin allergy due to zinc. *Lancet* 1:122–24, 1979
 8. Shore RN, Shelly WB, Kyle GC: Chronic urticaria from isophane insulin therapy. *Arch Dermatol* 11:94–97, 1975
 9. Schellekens P, Eijssvoogoz VP: Lymphocyte transformation in vitro. I. Tissue culture conditions and quantitative measurement. *Clin Exp Immunol* 3:571–78, 1968
 10. Lawrence RD: Local insulin reactions. *Lancet* 1:1125–26, 1925
 11. Bloom SR, Adrian TE, Barnes AJ, Polak JM: Autoimmunity in diabetics induced by hormonal contaminants of insulin. *Lancet* 1:14–16, 1979
 12. Bruni B, Gama S, Regis G, Turco GL: Proinsulin and A-component antibodies in diabetics after long term monocomponent insulin. *Diabetologia* 14:165–69, 1978
 13. Falholt K, Hoskam JAM, Karamanos BG, Süssstrunk H, Viswanathan M, Heding LG: Insulin-specific IgE in serum of 67 diabetic patients against human insulin (Novo), porcine insulin, and bovine insulin: four case reports. *Diabetes Care* 6 (Suppl. 1):61–65, 1983
 14. Grammer LE, Metzger BE, Patterson R: Cutaneous allergy to human (recombinant DNA) insulin. *JAMA* 251:1459–60, 1984
 15. Robbins DC, Shoelson SE, Tager HS, Mead PM, Gaynor DM: Products of therapeutic insulins in the blood of insulin-dependent (type I) diabetic patients. *Diabetes* 34:510–19, 1985
 16. Maislos M, Mead PM, Gayner DH, Robbins DC: The source of the circulating aggregate of insulin in type I diabetes is therapeutic insulin. *J Clin Invest* 77:717–23, 1986
 17. Robbins DC, Cooper SM, Fineberg SE, Mead PM: Antibodies to covalent aggregates of insulin in blood of insulin-using diabetic patients. *Diabetes* 36:838–41, 1987
 18. De Shazo RD, Boehm TM, Kumar D, Galloway JA: Dermal hypersensitivity reactions to insulin: correlations of three patterns to their histopathology. *J Allergy Clin Immunol* 69:229–37, 1982
 19. Blundell T, Dodson G, Hodgkin D, Mercola D: Insulin: the structure in the crystal and its reflection in chemistry and biology. *Adv Protein Chem* 26:279–402, 1972
 20. Brange J: *Galenics of Insulin*. New York, Springer-Verlag, 1987
 21. Pullen RA, Lindsay DG, Wood SP, Tickle IJ, Blundell TL, Wollmer A, Krail G, Brandenburg D, Zahn H, Glieman J, Gammeltoft S: Receptor-binding region of insulin. *Nature (Lond)* 259:369–73, 1976
 22. Pietri A, Raskin P: Cutaneous complications of chronic continuous subcutaneous insulin infusion therapy. *Diabetes Care* 4:624–26, 1981
 23. Saudek CD, Pitt HA, Dempsey M, Fischell RE, Rubio MS, Loman K, Wierski J: The programmable implantable medication system (PIMS): first human implantations (Abstract). *Diabetes* 36 (Suppl. 1):77A, 1987
 24. Loughheed WD, Woulfe-Flanagan H, Clement JR, Albisser AM: Insulin aggregation in artificial delivery systems. *Diabetologia* 19:1–9, 1980
 25. Loughheed W, Albisser AM, Martindale HM, Chow JC, Clement JR: Physical stability of insulin formulations. *Diabetes* 32:424–32, 1983