and there is no titer per se. By comparison, the iodinated Tg-T4 preparation for the same quantity produced a Δ 0–40 μg% of 40.6% and a titer of 750×. With this latter antiserum 35 pg of T4 could be detected. Cross-reactivity with T3 was only 7.1% (table 2), not limiting when considering the ratio of T3/T4 in human serum ($^1/_{50}$ to $^1/_{100}$). This early and intense response provided an antiserum with the required properties for direct use.

In the iodination 308 moles of I were bound per mole Tg. This figure is obtained by multiplying the % radiolabel bound × KI added (gravimetric correction for I in KI), converting to g and dividing by 126.9. The calculation of moles Tg reacted (mol.wt 669,000) then yields the ratio. Drawing an analogy with rat Tg for which published mole% amino acid composition data are available, one can estimate 100 residues of tyrosine per molecule (calculation based on prosthetic carbohydrate - less Tg) each taking up 2 atoms of I; thus over 100 moles I were available for the saturation iodination of lower-substituted thyronines; even with iodohystidyl formation (approximately 50 residues), the fact that some of the tyrosines would be present in iodinated form would compensate for the histidine participation, at least in part. The iodotyrosines are not immunocross-reactive with T4, that event requiring the aromatic ether linkage and B-ring iodination[4].

A discussion of the magnitude of thyroxylation is in order. A ratio of T4/ITg of 266 was obtained. There are an estimated 850–950 groups potentially reactive with thyroxine under these conditions; these figures are obtained from mole% composition data for aspartic and glutamic acids (include asparagine and glutamine) and lysine. However, there must certainly be some T4-T4 reactivity as well. One could possibly abrogate this inter-thyroxine effect by use of the N-acyl or alkyl ester derivatives or by using tetraiodothyroacetate or similar analogues.

A change in immunization procedure which could improve the T4-BSA method is worth discussing. When most of the inoculum mass is given simultaneously, followed by a single i.v. injection 4 months later, an improvement in titer with equivalent cross-reactivity is observed (table 3). It may also be possible to retain the improvements with shorter times before the i.v. challenge.

Table 2. Performance characteristics of antisera prepared with different types of T4 immunogens: early responses

Immunogen	Total dose (mg)	Titer	Δ0–40 μg** (%)	T3 cross-reactivity*** (%)
Iodinated Tg-T4	6.0	750	40.6	7.1
T4-BSA	6.0	nil*		

* Represents all findings from several determinations. ** Represents decrease in standard slope effected by 40 μg%. *** Represents the % reactivity of the antiserum with T3-^{125}I. Only minor changes in T4-^{125}I uptake were noted with the T4-BSA immunogen, while a significant titer and standard curve slope steepness were noted with the Iodinated Tg-T4 preparation; cross-reactivity with this immunogen was within acceptable limits. Injections of 2 mg were given on alternate weeks for 3 weeks; the preparations were given s.c. in complete Freund adjuvant.

Table 3. Simultaneous T4-BSA immunogen administration compared with sequential T4-BSA administration

Method	x̄ T4 titer (n = 4)	Range T3 cross-reactivity (%)	Δ0–40 μg (%)
a) Simultaneous	17,650	1.8–7.5	46.5
b) Sequential	7,000	1.9–6.3	>40.0

A gain of titer was noted with method (a) without sacrifice of specificity or avidity.

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Suppressed collagenolytic activity in polymorphonuclear leucocytes from diabetic humans[1]

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Summary. Extracts of polymorphonuclear leucocytes (PMNL) from diabetic humans exhibited less collagenolytic activity than extracts from normoglycemic control subjects. Partially purified control extracts produced α^A and α^B collagen breakdown products of the type generated by mammalian collagenase; the diabetic preparation produced decreased amounts of the same products. The diabetic PMNLs may synthesize abnormally low levels of collagenase or contain inactive forms of this enzyme.

Experimental diabetes in rats has been shown to increase the collagenolytic activity of gingival tissue in culture[3,4] and to result in collagen loss in skin[5] and bone[6]. In contrast, however, Golub et al.[7] found that diabetes suppressed the loss of collagen normally seen in an inflammatory gingival lesion. To explain this unexpected effect, the following were proposed: (a) that diabetes increased collagen cross-linking rendering the fibrils less susceptible to degradation by collagenase; experimental evidence now exists for this proposal,[8,9] and/or (b) that diabetes inhibited the activity of leucocytes which mediate collagen degradation during inflammation. Although several studies have demonstrated that diabetes inhibits various aspects of the inflammatory response[10,11], including leucocyte chemotaxis[12–14], the present study is the first to demonstrate an effect of diabetes on leucocyte collagenolytic activity.

Collagenolytic activity of polymorphonuclear leucocytes from control and diabetic human subjects*. Each reaction mixture, incubated for 14 h at 35 °C, contained 250 µl PMNL extract from 2.5×10^6 cells, from each subject, and 50 µl C^{14}-glycine labeled reconstituted collagen fibrils (9250 DPM)**

Experimental Group	% Collagen gel lysed	DPM C^{14}-gel released per µg DNA	2-h postprandial blood glucose (mg%)	Hemoglobin A_1 (%)
Control	$66.4 + 6^{aA}$	124.8 ± 12.2^{aA}	89.3 ± 5.7^{A}	8.8 ± 0.2^{a}
Control + 0.2 mM APMA	84.6 ∓ 6.1^{bA}	159.2 ± 16.5^{bA}		
Diabetic	28.1 ± 6.5^{B}	53.7 ± 14.4^{B}	277.0 ± 66.7^{B}	12.3 ± 2.1^{b}
Diabetic + 0.2 mM APMA	31.0 ± 7.6^{B}	57.2 ± 13.1^{B}		

* Each value represents the mean ± SEM for 5 subjects. ** 2.5×10^6 cells from the lymphocyte rich layer lysed 1.1% ± 0.6 of the C^{14}-collagen gel (control) and 1.3% ± 0.3 (diabetic). [a] vs [b], significantly different ($p < 0.05$); [A] vs [B], ($p < 0.01$).

Materials and methods. Heparinized whole blood, typically 20–30 ml per subject, was collected from control (non-diabetic) and diabetic male subjects 30–66 years of age. Aliquots were taken for glucose and glycosylated hemoglobin[15] analysis. The remainder was incubated at 37 °C (1 h) followed by centrifugation (400 × g, 15 min) to separate buffy coat and plasma. Contaminating erythrocytes were lysed by brief exposure to distilled water and NaCl was added in a final concentration of 0.85%. The leucocytes were collected by centrifugation, resuspended in plasma, and the PMNLs and mononuclear cells separated by Ficoll-Hypaque gradient centrifugation[16]. After 2 washes in saline, the cells were suspended in Earle's balanced salt solution (containing 5mM $CaCl_2$) at a concentration of 10^7 cells/ml (93–95% of the PMNLs were viable based on the Trypan blue exclusion technique).

To assay collagenolytic activity in the cell extract, the cells were ruptured by repeated freeze-thawing followed by brief homogenization at 4 °C. The PMNL and lymphocyte extracts were clarified by centrifugation (4 °C). The extract from each subject was assayed for collagenolytic activity individually, as described below. The extract's pellet was dissolved in 5 mM NaOH and assayed for DNA[17]. In some experiments, the PMNL extracts from each experimental group were pooled prior to measuring collagenolytic activity.

Collagenolytic activity was assayed using a modification[18] of the technique of Lazarus et al.[19]. The C^{14}-glycine labelled collagen gels were reacted with the crude enzyme preparations for 0.5–24 h at 35 °C, then filtered through a 0.8 µm pore size, 13 mm diameter, Metricel filter (Gelman) in a Swinney adaptor allowing the passage of solubilized collagen breakdown products. P-aminophenylmercuric acetate (APMA) was added to the reaction mixture in a final concentration of 0.2 mM to activate latent collagenase[20].

Collagenase in control and diabetic PMNL extract pools (from 10^7 cells/ml; see above) was partially purified with dioxane[21], dialyzed against 0.05 mM Tris-HCl (pH 7.6) containing 0.2 M NaCl and 5 mM $CaCl_2$, then concentrated 5-fold with lyphogel. 70 µl of the control or diabetic preparation was reacted with 50 µl C^{14}-collagen for 24 h at 20 °C in the presence of 0.7 mM APMA. The reaction mixture was heated to 60 °C for 30 min and the collagen breakdown products, generated by the control and diabetic

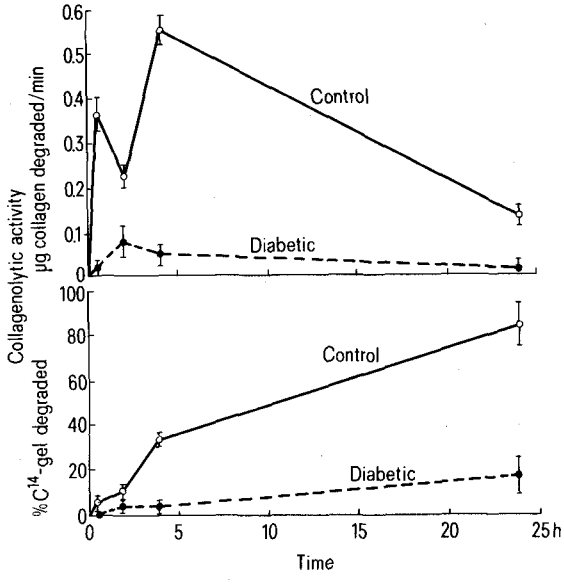

Fig. 1. Collagenolytic activity in extracts from 4 control (blood glucose = 76 ± 2 mg%; $HbA_1 = 7.5 \pm 0.5\%$) and 4 diabetic (blood glucose = 403 ± 68 mg%; $HbA_1 = 11.7 \pm 1.7\%$) human subjects. Reaction mixtures contained 50 µl C^{14}-collagen fibrils (13,820 dpm; 200 µg collagen) plus 200 µl PMNL extract from 2×10^6 cells and were incubated at 35 °C for 0.5–24 h.

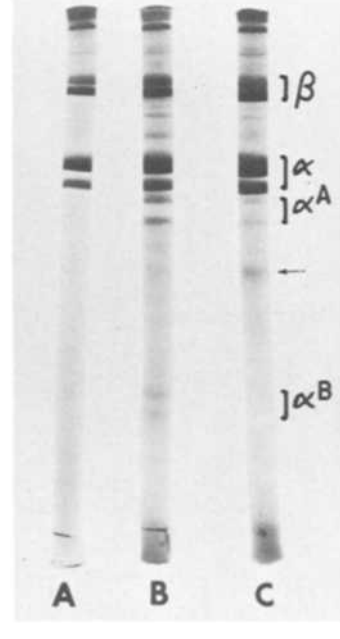

Fig. 2. SDS-acrylamide gel electrophoresis pattern of collagen breakdown products generated by partially purified collagenase from normal and diabetic PMNL. A = salt-soluble rat skin collagen; B = collagen plus normal PMNL enzyme; C = collagen plus diabetic enzyme.

preparations, were examined by polyacrylamide gel electrophoresis using the method of Yamada and Weston[22].

Results and discussion. Blank samples, lacking PMNL extract, solubilized 1.5±0.4% of the C^{14}-collagen gels; with APMA, blanks solubilized 0.8±0.1%. Trypsin, added to blank samples, degraded 6.5±1.2% of the C^{14}-gels reflecting the extent of non-specific protease-mediated collagen breakdown. Consistent with a previous study[19], the extracts from the lymphocyte-rich layer did not produce detectable collagenolytic activity (table).

In our initial experiments, control PMNL extracts from subjects with normal blood glucose and hemoglobin A_1 levels, degraded about 65% of the collagen fibrils after a 14-h incubation (table). In subsequent experiments, in which collagenolytic activity in extracts was assessed at several incubation times, the rate of collagen degradation appeared to reach a maximum at 4 h and was at its lowest level at 24 h when about 84% of the substrate had been consumed (figure 1). Treating the normal extracts with APMA increased collagenolytic activity about 28% (table). Individual PMNL extracts from the hyperglycemic diabetics showed 60% less collagenolytic activity, on an equivalent cell or DNA basis, than the controls (table). This was confirmed in the time study in which individual diabetic extracts showed 60–95% less activity at 0.5-, 2-, 4-, and 24-h incubation periods (figure 1). APMA treatment was not effective in normalizing the collagenolytic activity in the extracts of the diabetic leucocytes (table). Although higher concentrations of APMA (0.7 mM)[20] and pretreatment with trypsin[23] increased collagenase activity in the leucocyte extracts from the normal subjects, these treatments had no effect on the activity of the extracts from the diabetic patients (data not shown).

The partially purified control and diabetic extracts produced collagen breakdown products of the type generated by mammalian collagenase (figure 2). The control preparation produced α^A and α^B products with molecular weights $^3/_4$ and $^1/_4$ that of the α chains, calculated from a Weber and Osborne standard curve[24]. As expected from the above data, the diabetic extract generated reduced amounts of α^A and α^B. The diabetic gel exhibited a band migrating between α^A and α^B which was not seen on the normal gel. After slicing a diabetic gel containing C^{14}-collagen subunits, and counting the fractions in a liquid scintillation spectrometer, this band was found to contain little, if any, C^{14}-glycine, and, therefore, did not consist of non-specific collagen breakdown products. We are currently determining whether this material is a collagenase inhibitor present in abnormally high levels in the diabetic cell.

At the present time, it is not clear whether the observed deficiency in the leucocytes of the diabetics reflects decreased synthesis and storage of collagenolytic enzyme(s) or impaired activity of a normal amount of collagenase possibly due to the presence of excessive amounts of enzyme inhibitor. These possible mechanisms are being examined in our laboratory.

Leucocyte dysfunctions in diabetics include impaired adherence[25], chemotaxis[12–14], phagocytosis[26], and intracellular microbicidal activity[27]. These defects explain, in part, their increased susceptibility to infection[28]. Diabetes can also affect the progress of wound healing[29]. In tissues such as skin, damaged connective tissue must be resorbed before re-epithelialization can be completed[30]. PMNLs play a role in the wound healing process by releasing collagenase and other proteolytic enzymes necessary for debridement[30]. A reduced ability of diabetic PMNLs to degrade interstitial collagen could impair debridement and, as a result, wound healing. An inability of these cells to degrade type IV collagen, if found, could impair their movement through basement membranes of blood vessels thus contributing to the suppressed acute inflammatory response[10]. We are currently examining the activity of elastase in diabetic human leucocytes since this neutral protease, rather than collagenase, appears to be responsible for the degradation of basement membrane collagen[31].

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