n = 5). Consequently, it appears that the factor present in the bursa copulatrix acts to suppress the action of PBAN very rapidly but does not alter the activity of PBAN.

It has been reported that the titer of pheromone drops significantly during mating due to the transfer of a male-produced factor but that the action of this 'male factor' is short-lived and can be overcome by restimulation with PBAN[15]. Nonetheless, mated females produce less pheromone than their virgin counterparts for several days after mating[7]. We have found that injection of PBAN plus the extract of the bursa of young females (3rd photophase) sampled on the day after mating resulted in the production of only 3.1 ng (± 0.9, n = 5) of Z11–16:AL whereas 34.0 ng (± 3.8, n = 5) of Z11–16:AL were produced when extracts of the bursa obtained from virgin females of the same age plus PBAN were injected. Therefore, we believe that under natural field conditions, production and release of this factor we have termed 'pheromone biosynthesis suppression factor' (PBSF), are stimulated by mating. Such release of PBSF may result in the maintenance of the much reduced pheromone titer present in the glands of mated females for several days, thereby rendering them unattractive to males. Endogenous regulation of this type is advantageous to moth species because it reduces competition between mated and virgin females, thus increasing the probability that virgins will mate and thereby increasing genetic diversity. The refractory period after mating, when pheromone titer is very low, would allow females to lay fertilized eggs without threat of being accosted by sexually aroused males. Furthermore, the inhibition of pheromone production among senescing females, be they virgin or mated, would be advantageous to males because males would not be attracted to and would not waste reproductive effort and energy on females likely to die before laying fertilized eggs.

Although data reported here are for *H. zea*, we have also found that this factor suppresses pheromone production in several other noctuid moth species including *H. subflexa*, *H. virescens*, and *Spodoptera frugiperda*. Additionally, we have evidence that a similar substance is produced by females of a sphyngid moth, the tobacco hornworm, thus suggesting that the factor is produced by a large number of moths from several families.

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Effect of recombinant human granulocyte colony-stimulating factor on human neutrophil adherence in vitro

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Summary. We measured the effects of recombinant human granulocyte-colony stimulating factor (rhG-CSF) on the adherence of human neutrophils by using a dacron fiber system to assay the adhesive ability of neutrophils. rhG-CSF enhanced neutrophil adherence to dacron fibers. N-formyl-methionyl-leucyl-phenylalanine (fMLP) induced neutrophil-neutrophil interaction (neutrophil aggregation) in addition to neutrophil-dacron interaction, whereas rhG-CSF did not cause neutrophil aggregation. These results indicated that rhG-CSF increases the adhesive ability of neutrophils without neutrophil-neutrophil interaction, and the action of rhG-CSF in neutrophil activation is different from the neutrophil activation caused by fMLP.
Key words. Recombinant human G-CSF; neutrophil; adherence; dacron.

G-CSF stimulates the growth of progenitor cells of neutrophils[1,2]. Human G-CSF was isolated from the human bladder carcinoma cell line 5637[3], and was cloned and expressed in *E. coli*[4]. Recombinant human G-CSF (rhG-CSF) was shown to increase the peripheral blood neutrophil count in humans[5,6]. G-CSF binds to specific

receptors on mature human neutrophils as well as the progenitor cells[7,8] and augments mature neutrophil functions, oxidative metabolism[9–11], chemotaxis[12], or survival[13] in vitro. rhG-CSF has a potent effect on neutropenic host defense against infection[14,15], and the protective effect of rhG-CSF is ascribed to an increase in neutrophil counts and enhanced functions. In particular, adherence of circulating neutrophils to the endothelium is the first stage of neutrophil migration and is very important for host resistance. Furthermore, the alteration of the adhesive ability of neutrophils influences their kinetics in the circulation. However, little has been reported about the effects of rhG-CSF on the adhesive ability of neutrophils. We therefore evaluated the effects of rhG-CSF on neutrophil adherence by using a simple new method, the dacron fiber assay.

Materials and methods

rhG-CSF. rhG-CSF, a protein of MW 18,799, was produced by *E. coli* and purified. It had a specific activity of 1.5×10^8 U/mg protein, as described by Tanaka et al.[16]. A test solution of rhG-CSF was prepared in sodium acetate buffer.

Reagents. N-formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma Chemical), epinephrine (Sigma Chemical) and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS, Dojin Chemical Laboratory) were used for this study.

Neutrophil adherence assay. Neutrophils were isolated from heparinized venous blood from healthy volunteers using Mono-Poly Resolving Medium (Flow Laboratories). Purity and survival of the isolated neutrophils was 98% and 99%, respectively. The neutrophils were suspended in 10% fetal calf serum-Hanks' balanced salt solution (HBSS) to a final cell concentration of 5×10^6 cells/ml.

Adherence of neutrophils was measured by determining the numbers of neutrophils, in the purified neutrophil suspension or in whole blood, that passed through a dacron fiber (1.3 denier) column. Each column was prepared by packing 100 mg dacron fiber into a 0.5-ml volume of a disposable syringe for the whole blood and 30, 50 or 70 mg of dacron fiber into a 0.15, 0.25 or 0.35-ml volume, respectively, for the neutrophil suspension. 1 ml of the neutrophil suspension, or heparinized (10 units/ml) whole human blood, was preincubated with rhG-CSF, its vehicle, or the other reagents for 10 min at 37 °C. The original samples were passed through the column, which was placed in an incubator at 37 °C at high humidity. Total leukocyte counts and differential counts of effluent and original samples were measured using an auto cell counter (E-2000, Toa Medical Electronics Co.) and an auto cell analyzer (MICROX, Tateishi Electronics), respectively. The percentage of adhesive neutrophils was calculated from the following equation:

$$\text{Adherence (\%)} = 100 - \frac{\text{neutrophils/ml in effluent sample}}{\text{neutrophils/ml in original sample}} \times 100$$

Measurement of neutrophil aggregation. Isolation of neutrophils from healthy volunteers was carried out in the same way as in the adherence assay method. The neutrophils were suspended in 0.5% bovine serum albumin-HBSS to a final cell concentration of 1.5×10^7 cells/ml. The neutrophils were preincubated at 37 °C and aggregation was measured using a platelet aggregometer (HEMA TRACER 1 model PAT-6M, Niko Bioscience Inc.). Aggregation in response to fMLP (1 μM) or rhG-CSF (10 or 100 ng/ml) was recorded as increased light transmission.

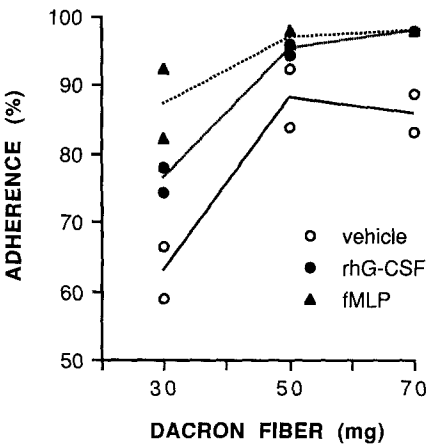

Figure 1. Effect of rhG-CSF on neutrophil adherence to dacron fibers. Purified neutrophils were preincubated with rhG-CSF vehicle, rhG-CSF or fMLP for 10 min at 37 °C. The adherence of neutrophils was measured by a dacron fiber (30, 50 or 70 mg) assay. Data represent the means of duplicates.

Effect of rhG-CSF on adherence (%) of neutrophils

	Control (vehicle)	rhG-CSF (20 ng/ml)	rhG-CSF (100 ng/ml)	rhG-CSF (500 ng/ml)	fMLP (1 μM)	Epinephrine (10 μg/ml)	rhG-CSF + DIDS (100 ng/ml) (250 μg/ml)	fMLP + DIDS (1 μM) (250 μg/ml)
Exp. 1	69.7 ± 4.1			91.5 ± 2.0[a]	98.7 ± 0.7[b]			
Exp. 2	54.9 ± 2.9	94.5 ± 2.1[b]	95.7 ± 1.1[b]		99.3 ± 0.4[b]			
Exp. 3	77.1 ± 2.1		98.3 ± 0.6[b]			63.7 ± 1.3[a]	98.6 ± 0.8[b]	
Exp. 4	82.0 ± 2.3				99.7 ± 0.1[a]			99.9 ± 0.2[a]

Heparinized whole human blood was preincubated with rhG-CSF vehicle, rhG-CSF, fMLP, or epinephrine for 10 min at 37 °C. DIDS was added 5 min before the addition of rhG-CSF to blood samples. The adherence (%) of neutrophils was measured by the dacron fiber assay. The data represent the means of four samples ± SE. [a] $p < 0.01$; [b] $p < 0.001$; Significantly different from control group.

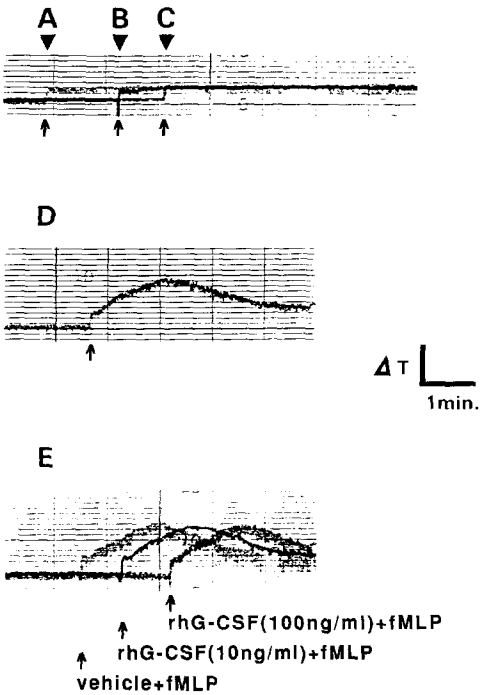

Figure 2. Effect of rhG-CSF on neutrophils aggregation. Neutrophil aggregation was recorded as increased light transmission (ΔT, y-axis) over a period of time shown in minutes (x-axis). Lines A, B, C, and D show responses to vehicle of rhG-CSF, 10 ng/ml rhG-CSF, 100 ng/ml rhG-CSF, and 1 μM fMLP, respectively. Line E shows responses to 1 μM fMLP at 10 min after the addition of rhG-CSF (10, 100 ng/ml) or vehicle. Arrows indicate the point at which the stimulus was added to the cell suspension.

Statistical analysis. Results were statistically evaluated using Student's t-test.

Results

The change in adherence of neutrophils after incubation of whole blood with rhG-CSF, fMLP, or epinephrine is shown in that table. rhG-CSF (20–500 ng/ml) or fMLP (1 μM) markedly enhanced the adherence of neutrophils. rhG-CSF or fMLP-induced neutrophil adherence was not influenced by preincubation with DIDS (250 μg/ml). Conversely, epinephrine (10 μg/ml) significantly inhibited the adherence of neutrophils. In experiments using purified neutrophils, an increase in adherence was observed which was dependent on dacron volume, and rhG-CSF or fMLP enhanced this adherence (fig. 1). Figure 2 shows the effect of rhG-CSF on neutrophil aggregation. rhG-CSF (10–100 ng/ml) did not induce neutrophil aggregation, unlike fMLP (1 μM), and did not affect fMLP-induced neutrophil aggregation.

Discussion

Neutrophils are phagocytic cells which play a vital role in host resistance and inflammation. Neutrophils have various functions and one of these is adherence. In this study, rhG-CSF enhanced the adherence of neutrophils to dacron fibers, and did not cause neutrophil aggregation, which is a disadvantageous effect for the host. However, fMLP induced both neutrophil adherence and neutrophil aggregation. fMLP is known to increase neutrophil membrane surface expression of CD11b/CD18, which is related to aggregation[17]. rhG-CSF may not induce the expression of surface glycoproteins because rhG-CSF did not cause neutrophil aggregation, and neutrophil adherence to artificial surface is not dependent on up-regulation of CD11b/CD18[18]. Moreover, rhG-CSF-induced neutrophil adherence was not inhibit by DIDS. DIDS, an anion channel blocker, inhibits the increase in surface expression of CD11b and neutrophil aggregation[19]. We suggest that rhG-CSF enhances neutrophil adherence to dacron fibers without increasing the expression of the surface glycoprotein and that neutrophil adherence is due to other mechanisms such as changes in the electrical charge of the neutrophil surface membrane[20].

Intravenous infusion of fMLP or epinephrine into animals induces acute neutrophil margination[21] or demargination[22], respectively. Administration of rhG-CSF into humans also induces an immediate fall in circulating neutrophil count[5,6]. Thus, the results of this in vitro assay correspond to the in vivo effects. The reason for the acute margination is not clear, but it is possible that electrical charges of the surface membrane of neutrophil are changed[23] or neutrophil membrane rigidity is increased[24]. The detailed mechanisms of neutrophil adherence induced by rhG-CSF and the relation between adherence to dacron and margination of neutrophils requires further investigation. Our findings clearly demonstrate that rhG-CSF increases the adhesive ability of neutrophils in the dacron fiber assay system.

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In vitro culture of chick down feather bulbi: A tool to obtain proliferating and differentiating keratinocytes in an organotypic structure

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Summary. Chick down feather bulbi can be cultured in different culture systems. Morphological analysis and ^{3}H-thymidine incorporation measurements prove that the majority of cells are viable epithelial cells.
Key words. Organotypical cultures; feather bulbus; keratinocytes.

The majority of cells in organotypic skin cultures are fibroblasts. Using cultures of embryonic chick skin fragments, we previously studied the effects of drugs on the fibroblasts, and to a lesser extent also on the epithelial cells in their interaction with fibroblasts[1,2].

We looked for an experimental model with a population consisting predominantly of epithelial cells, in order to evaluate immediate effects of chemical and physical agents on keratinocytes. Starting from down feather bulbi of a 13.5-day-old chick embryo, we have developed an experimental model which fulfils the criteria mentioned above[3]. The developing rachis and the associated barb system[4] of the bulbus both consist of cells of epithelial origin. These structures are covered with the follicle sheath, a layer of keratin. Fibroblasts are present only in the central pulp region.

As staging is easy in embryonic chicks[5], reproducible bulbi can be prepared and explanted in vitro.

Description of the technique (fig. 1)

Preculture and preparation of the bulbi

Down feathers of the dorsal region of 13.5-day-old chick embryos (white leghorn) were epilated with forceps and collected in Ringer's physiological solution. Intact feathers normally show a proximal dilatation, representing the bulbar region[4]. Feathers which showed this proximal dilatation were selected under a dissecting microscope and transferred into a glass petri dish containing MEM with Hanks' salts, 10% fetal calf serum and antibiotics. At this stage it was impossible to isolate the bulbar region from the shaft of the feather in a reproducible way.

When the feathers were precultured for 5 days at 37 °C, the bulbus could be easily isolated from the shaft. The proximal part of the feather enlarged to form a rounded-up bulbar region during this culture period. The separation of the bulbus was achieved with a watchmaker's needle under a dissection microscope. Bulbi with a diameter of 0.5 mm $\pm$ 0.1 mm were selected. These bulbi will be referred to as precultured bulbi.

Methods for culturing the precultured bulbi

Depending on the aim of the study, the bulbi were incubated either a) on the bottom of a plastic tissue culture flask, or b) on top of a semisolid medium, or c) in a glass tissue-culture flask on a gyratory shaker (fig. 1).

The culture medium used in the different culture systems was MEM with Hanks' salts (when using plastic flasks or semisolid medium) or with Earle's salts (in glass flasks), supplemented with 10% FCS and antibiotics.

a) *Culture on a plastic surface.* Some precultured bulbi were explanted on the bottom of a plastic tissue culture flask, submerged in fluid medium and incubated at 37 °C. They were fixed and stained in toto after different periods of culture. Using this method of culture, the outgrowing cells of the attached bulbi could be examined with the light microscope.

b) *Culture on a semisolid medium.* Semisolid medium was made by mixing the culture medium with premelted agar at a concentration of 0.5%.

Some precultured bulbi were placed on this semisolid medium and incubated at 37 °C for different periods. After fixation, the fragments were prepared for light and electron microscopy. Using this culture method, the ar-