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Different kinds of polypeptides and polypeptide-coated nanoparticles are accepted by the selective transcytosis shown in the rabbit nasal mucosa

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Abstract

The specific transcytosis of polypeptides, demonstrated in the nasal respiratory mucosa of the rabbit, seems to be involved in antigen sampling at the airway entry, since absorption has been shown only to occur if lymphoid aggregates are present beneath the epithelium and to be proportional to aggregate volume. Nanoparticles and many polypeptides besides the two previously tested (i.e. carbocalcitonin (CCT) and adrenocorticotropic hormone) should be transportable, in agreement with the vesicular transcytosis and antigen sampling hypothesis. Thus unidirectional mucosa-submucosa and opposite fluxes (J_{ms}, J_{sm}) and the corresponding net fluxes (J_{net}) of uncoated or polypeptide-coated polystyrene nanospheres (diameter: about 0.5 μm) have been measured with the aid of spectrophotometry and quantitative dark-field microscopy. No net transport has been observed for uncoated beads, whereas it has always been shown for polypeptide-coated beads, although to different extents. The selectivity sequence for the polypeptides tested is as follows: BSA ≅ enkephalin ≪ anti-BSA IgG ≅ IgA ≅ CCT ≅ insulin ≤ anti-insulin IgG. With the exception of BSA and enkephalin-coated beads, whose J_{net} is very small, in all the other cases the apparent affinities for receptors seem to be equal or similar; just over 6% polypeptide coating on the nanosphere is sufficient to elicit maximal transport; finally, transport seems to require many cooperating binding sites between the single nanosphere and receptors or one or many non-cooperating binding sites, but with a threshold number of polypeptide molecules adsorbed on the nanosphere to reach a minimal binding probability.

Keywords: Active transport of polypeptides; Antigen; Antibody; Upper concha; Insulin; Carbocalcitonin

1. Introduction

Transepithelial active absorption of carbocalcitonin (CCT) and adrenocorticotropic hormone (ACTH) has been shown to be present across the respiratory mucosa covering the upper nasal concha of the rabbit [1]. The transport has been demonstrated to be supported by receptor-mediated endocytosis and transcytosis across the epithelium [2–4] and to be preclusively correlated and proportional to the total volume of lymphoid cell aggregates in the mucosa [5]. On this basis it has been concluded that the function of this transcytosis of polypeptides may be antigen sampling from the nasal lumen at the airway entry [5].

This paper and the following one examine the anti-

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gen sampling hypothesis further. In line with it we show in this paper that many different polypeptides besides CCT and ACTH can be selectively recognized by receptors and transcytosed; they have been presented adsorbed on nanoparticles, as the CCT, adsorbed on nanoparticles, has been previously observed to be equally transported in agreement with vesicular transcytosis [3]. In the following paper, using polypeptide-covered fluorescent nanoparticles, we also report that the transcytosis is localized in limited regions of the epithelium, lying on lymphoid aggregates, and that the epithelial cells involved are non-ciliated microvillar cells.

2. Materials and methods

Male New Zealand rabbits, weighing approx. 3 kg, were killed by cervical dislocation and the nasal mucosae from the roof of both nostrils were mechanically excised very carefully, washed with Krebs-Henseleit saline at room temperature and mounted on frames between two Teflon chambers. The exposed part of the mucosa (area: 0.3 cm^2) corresponded to that covering the upper concha. The two chambers were filled with 1 ml Krebs-Henseleit saline, bubbled with pre-humidified 95% O_2+5% CO_2 to oxygenate the tissue, maintain the pH at 7.4 and stir the solution. The experiments were carried out at a monitored temperature of 27 ± 1°C unless otherwise indicated; at this temperature the short-circuit current, index of ion transports, was half that measured at 37°C, but the isolated mucosa was stable and viable for hours [6]. The viability of the epithelium was checked at the beginning, during and at the end of each experiment by measuring the transepithelial electrical potential difference, as previously reported [1,6].

2.1. Flux measurements

The unidirectional mucosa-submucosa and opposite transports of native or polypeptide-covered latex nanospheres were determined for 2 h, one on the right and the other on the left mucosa from the same animal (with random combinations), with the protocol previously described [3]. Transports were expressed as fluxes (nanospheres transported cm^{-2} h^{-1}: nan cm^{-2} h^{-1}). Both fluxes were considered unidirectional since the maximal concentration of nanospheres reached in the flux chamber was 10^{-4}–10^{-5} compared to that applied in the opposite donor chamber. The bead concentration in the donor chamber, measured on 10 μl samples taken at the beginning (1 min after contact with the tissue) and at the end of the 2 h experiment, proved not to be significantly different in any case (initial concentration: $(3.30 \pm 0.03) \times 10^{11}$ nanospheres/ml, $n = 172$; final concentration: $(3.25 \pm 0.03) \times 10^{11}$ nanospheres/ml, $n = 172$).

The latex nanoparticles were 'polybead polystyrene microspheres' (nominal diameter: 0.5 μm; actual diameter of the lots used: 0.480–0.499 μm) produced by Polysciences (Warrington, PA, USA). For the concentration measurements a Lambda 5 spectrophotometer (Perkin Elmer, Norwalk, CT, USA) was used (wavelength = 600 nm). To prepare the calibration straight line the reference bead concentrations were predetermined by a Burker's chamber after adequate dilution. The nanospheres transported in the 2 h experiment were measured exclusively by Burker's chamber due to their very low concentration. All determinations in Burker's chamber were carried out under observation with an Eclipse E600 dark-field microscope (Nikon Europe, Badhoevedorp, The Netherlands) at a 400× magnification, taking advantage of Tyndall's effect. With a Burker's chamber obtained in a 1.5 mm thick glass slide, the ratio between the measured and calculated value of the nanospheres was 1.

The polypeptides were adsorbed on nanoparticles by a method previously set up [3,7]. Polypeptide concentration in the medium used for the adsorption process was 6.5×10^{-6} M (unless otherwise specified). Still unbound surface sites were completely blocked with bovine serum albumin (BSA) [3,7].

In one set of experiments the beads used were not covered with polypeptide. Under these conditions, when placed in the Krebs-Henseleit saline, they tend to adsorb and aggregate, so the donor saline and saline with transported beads were sonicated at the beginning and end of the experiment, just before carrying out the determinations. Moreover, the donor saline was completely withdrawn and renewed every 30 min, as this operation proved sufficient to maintain a constant free bead concentration.

2.2. Protein determination, materials and bathing salines

The protein covering of the nanospheres was only evaluated for insulin by measuring the protein in the covering incubation medium before and after incubation with nanospheres. The Bradford method (microassay; measurement range: 1–25 μg/ml protein) was used to determine the proteins [8].

BSA, purified human colostral immunoglobulin A (IgA), murine monoclonal anti-human insulin immunoglobulin G_1 and anti-BSA immunoglobulin G_2 (anti-ins. IgG and anti-BSA IgG) were supplied by Sigma (St. Louis, MO, USA) and bovine Na-insulin and enkephalin ([Leu 5]enkephalin) by Calbiochem (AG Luzern, Switzerland). Carbocalcitonin ([1,7 Asu]eel calcitonin or elcatonin, CCT) was donated by ISF-Biomedical Research Laboratories (Trezzano, Milan, Italy).

The Krebs-Henseleit solution had the following composition (mM): 142.9 Na^+, 5.9 K^+, 2.5 Ca^{2+}, 1.2 Mg^{2+}, 127.7 Cl^-, 24.9 HCO_3^-, 1.2 SO_4^{2-}, 1.2 $H_2PO_4^-$, 5.5 glucose; pH 7.4. The concentration of the polypeptides added to the Krebs-Henseleit saline was always molal, unless otherwise specified.

2.3. Statistics

The results are expressed as means ± standard errors (S.E.). Student's *t*-test for unpaired or, when possible, for paired data was used for statistical analysis. Interpolating sigmoidal or hyperbolic curves were calculated by non-linear regressions.

3. Results

3.1. Transepithelial fluxes of native or polypeptide-coated nanospheres

Table 1 shows that uncoated nanospheres do not display any significant net flux across the respiratory mucosa of the upper concha, in agreement with a previous observation [3]. Conversely, BSA-coated nanospheres (BSA-cn, BSA MW = 66 000) display a relatively very small but highly significant net absorption of about 2×10^6 nan cm^{-2} h^{-1}. The BSA coating was apparently partial as it occurred in the presence of only 6.5×10^{-6} M BSA (0.043 g%, w/v); however, the subsequent washings with 5% BSA (w/v), to block all still unbound surface sites, completely covered the nanospheres with BSA. The affinity of receptors to BSA should therefore be very slight. The equally small and significant net flux of enkephalin-coated nanospheres (enk-cn), which does not appear significantly different from that of BSA-cn, can be originated either by the enkephalin adsorbed (in the presence of 6.5×10^{-6} M

Table 1
Transepithelial fluxes of native or polypeptide-covered nanospheres

Covering	*n*	J_{ms}	J_{sm}	J_{net}
Uncovered	5	3.94 ± 0.48	3.37 ± 0.44	0.50 ± 0.38
Covered with				
1. BSA	11	6.02 ± 0.60"	4.15 ± 0.30	1.87 ± 0.38**
2. enkephalin	6	7.26 ± 0.53"	4.66 ± 0.09	2.60 ± 0.53**
3. carbocalcitonin	6	12.10 ± 1.40"	5.80 ± 0.80	6.30 ± 1.20**
4. insulin	30	10.56 ± 0.70"	3.45 ± 0.29	7.10 ± 0.57**
5. IgA	9	9.92 ± 0.97"	3.83 ± 0.11	6.10 ± 0.91**
6. anti-BSA IgG	6	10.15 ± 1.24"	4.08 ± 0.34	6.06 ± 0.94**
7. anti-insulin IgG	13	12.69 ± 1.06"	3.86 ± 0.26	8.83 ± 0.88**

After 30 min preincubation, 3.3×10^{11}/ml native or polypeptide-covered nanospheres (polystyrene, 0.5 μm diameter) were added to the mucosal or submucosal compartment, and the overall corresponding unidirectional transports during a 2 h period were determined by a Burker chamber and expressed as equivalent fluxes (J_{ms}, J_{sm}; 10^6 nan cm^{-2} h^{-1}); the net flux (J_{net}) was calculated from their difference. Bead covering was carried out by incubating the nanospheres with the 6.5×10^{-6} M polypeptide at 37°C for 90 min. The results are presented as means ± S.E.; *n*, number of experiments.

"$P < 0.01$ compared with the corresponding J_{sm}.

**$P < 0.01$ compared with zero.

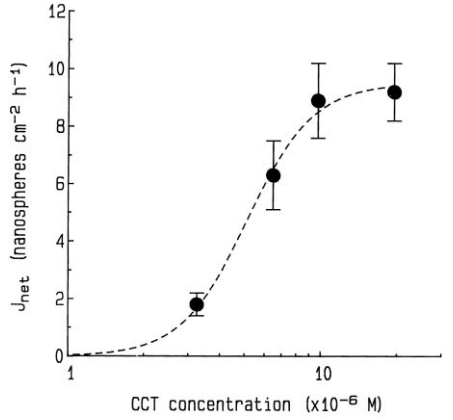

Fig. 1. Net fluxes of CCT-coated nanospheres as a function of CCT concentration in the covering medium and, as a consequence, of CCT adsorbed on nanospheres. Data points, presented as means ± S.E. ($n = 6$ for each data point), correspond to the J_{net} values reported in Table 2. The interpolating sigmoidal curve (dashed line) has been calculated on the basis of these data.

enkephalin) or by the BSA equally adsorbed (during washings).

Coating with polypeptides with a higher relative molecular weight than enkephalin (MW = 555.6), such as carbocalcitonin (MW = 3362), insulin (MW = 6000), monomeric anti-BSA or anti-insulin IgG (MW = 146 000) or dimeric secretory IgA (MW = 385 000), significantly increases the net flux to 6–9 × 10^6 nan cm^{-2} h^{-1} ($P < 0.01$ compared both with uncoated and BSA-coated nanospheres). Thus these polypeptides should be recognized by receptors much more than BSA, all the more so because they are present with fewer molecules adsorbed than BSA (in fact at the concentration used in the covering medium, i.e. 6.5 × 10^{-6} M, these polypeptides should block only a small number of adsorption sites: see further insulin adsorption as a function of its concentration). Finally it should be emphasized that anti-insulin IgG-cn exhibit a net flux significantly greater than that of anti-BSA IgG-cn or IgA-cn ($P \leq 0.05$).

Unlike the mucosa-submucosa unidirectional fluxes (J_{ms}), whose values largely depend on the coating kind, the opposite unidirectional fluxes (J_{sm}) display values generally independent of the coating, as they are not significantly different from one another and fluctuate in the narrow range of 3–4 × 10^6 nan cm^{-2} h^{-1} (uncovered beads or beads covered with BSA, insulin, IgA, anti-BSA IgG, anti-insulin IgG). Only in the case of enkephalin and carbocalcitonin do J_{sm} values fluctuate in the small (but significant) higher range of 4–6 × 10^6 nan cm^{-2} h^{-1}.

3.2. Net absorption of carbocalcitonin-coated nanospheres

In the case of CCT-cn, fluxes of beads coated at different concentrations of the polypeptide in the covering medium were also measured (Table 2); the nanosphere concentration was that usually fixed (3.3 × 10^6 nan/ml). As a consequence of this procedure, different CCT amounts should be adsorbed per nanosphere. Under these conditions the submucosa-mucosa unidirectional flux (J_{sm}) was unaffected: J_{sm} was not significantly different at the different coating concentrations, in line with the observation that J_{sm} does not depend on or is only slightly affected by coating (Section 3.1). Conversely, the mucosa-submucosa unidirectional flux (J_{ms}) and the net flux (J_{net}) increased with coating CCT con-

Table 2
Transepithelial fluxes of nanospheres covered with CCT at different concentrations

CCT concentration (M)	n	J_{ms}	J_{sm}	J_{net}
a. 3.2 × 10^{-6}	6	6.5 ± 0.6*	4.7 ± 0.6	1.8 ± 0.4**
b. 6.5 × 10^{-6}	6	12.1 ± 1.4**	5.8 ± 0.8	6.3 ± 1.2**
c. 9.7 × 10^{-6}	6	13.6 ± 1.3**	4.7 ± 0.4	8.9 ± 1.3**
d. 19.5 × 10^{-6}	6	15.2 ± 1.2**	6.0 ± 0.5	9.2 ± 1.0**

Native nanospheres (3.3 × 10^{11} nan/ml) were exposed to CCT at different concentrations, at 37°C for 90 min. Then the mucosa-submucosa and opposite transepithelial fluxes (J_{ms}, J_{sm}) and their difference (J_{net}) were measured by the usual procedures at 27°C. Fluxes are expressed as 10^6 nan cm^{-2} h^{-1}. Results are reported as means ± S.E.; n, number of experiments.

*, **$P < 0.05$ or 0.01 compared with the corresponding J_{sm}.

**$P < 0.01$ compared with zero.

centrations to reach a maximum at about 10 μM (Table 2).

By plotting net fluxes versus coating CCT concentrations (Fig. 1), the best fit for data points is obtained with a sigmoidal curve whose $S_{0.5}$ is 5.1 ± 1.1 μM, J_{max} (9.3 ± 0.7)×10^6 nan cm^{-2} h^{-1}, reached at about 10–15 μM CCT; the Hill number is 3.3; $r^2 = 0.986$ ($P < 0.01$). At the reference concentration of 6.5 μM CCT, J_{net} is about 68% J_{max}. Alternatively, data points can be interpolated by a hyperbola ($r^2 = 0.961$; $P < 0.01$), with $K_m = 4.6 \pm 09$ μM and $J_{max} = (10.2 \pm 1.2) \times 10^6$ nan cm^{-2} h^{-1}; however, to have an r^2 comparable with that obtained with the sigmoid, the hyperbola calculated intercepts the x-axis at a threshold concentration (approx. 3 μM) below which transport is not activated.

3.3. Net absorption of insulin-coated nanospheres

Nanosphere coating with insulin and fluxes of insulin-cn have been examined in much more detail.

First, the net flux observed with nanospheres covered at the reference concentration of 6.5×10^{-6} M insulin was studied in relation to its metabolic dependence. Table 3 demonstrates that J_{ms} is about twice as large at 37°C as at 27°C ($P < 0.05$); J_{sm} is not significantly affected, in agreement with a passive nature; J_{net} displays a thermic coefficient Q_{10} equal to 2.2, in agreement with metabolic dependence. Table 3 also reports that, by treating the tissue with metabolic inhibitors (3×10^{-3} M monoiodoacetate+10^{-4} M dinitrophenol), net flux is reduced to a level not significantly different from zero.

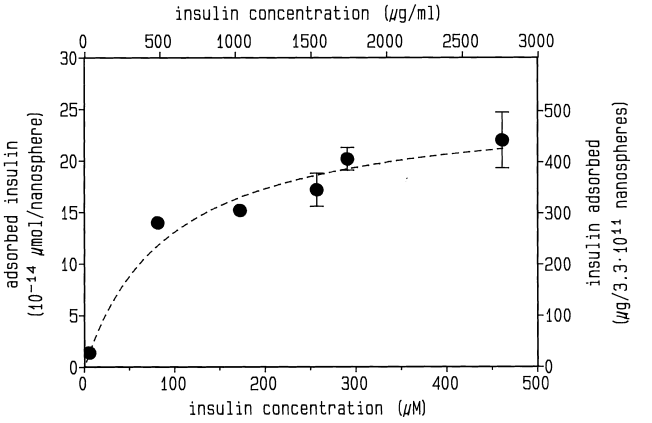

Fig. 2. Insulin adsorbed on nanospheres as a function of insulin concentration in the covering medium. The covering medium (Krebs-Henseleit saline) contained insulin at different concentrations; the nanosphere concentration was 3.3×10^{11} nan/ml; incubation was performed for 90 min at 37°C as usual. The insulin in the medium was measured before and after the covering incubation; the insulin remaining adsorbed on the beads was calculated from the difference of these two determinations. The ordinate on the left indicates the μmoles of insulin adsorbed per nanosphere as a function of the μM insulin concentration in the medium (lower abscissa); the ordinate on the right indicates the total μg of insulin adsorbed on all the nanospheres present in the medium as a function of μg/ml insulin in the medium (upper abscissa). The data points are the means ± S.E. of the results of four separate covering experiments. The dashed line represents the interpolating hyperbola.

Second, insulin adsorbed on nanospheres as a function of insulin concentration in the covering medium has been measured. Fig. 2 shows the results: the insulin adsorbed increases with hyperbolic kinetics. The kinetics analysis shows that the maximal amount of insulin adsorbed per nanosphere is equal

Table 3
Effect of temperature, metabolic inhibitors and insulin concentration on transepithelial fluxes of insulin-covered nanospheres

Experiment conditions	n	J_{ms}	J_{sm}	J_{net}
a. 27°C	13	11.06 ± 1.47"	3.96 ± 0.57	7.10 ± 1.15**
b. 37°C	4	20.90 ± 3.60"	4.20 ± 0.60	16.70 ± 3.10**
c. MIA+DNP	8	8.69 ± 2.16"	7.12 ± 2.04	1.57 ± 2.07
d. Covering with 4.7×10^{-4} M insulin	6	13.80 ± 2.31"	4.01 ± 0.22	9.80 ± 2.12**,ns

Under experiment conditions a, b and c bead covering was carried out with 6.5×10^{-6} M insulin at 37°C for 90 min; under condition d, the insulin concentration during covering was increased to 4.7×10^{-4} M; under all conditions, the bead concentration was 3.3×10^{11} nan/ml. The transepithelial fluxes (J_{ms}, mucosa-submucosa; J_{sm}, submucosa-mucosa; J_{net}, $J_{ms} - J_{sm}$) were measured by the usual procedures, at 27°C, with the exception of condition b (37°C). The fluxes are expressed as 10^6 nan cm^{-2} h^{-1}. Monoiodoacetate (MIA) = 3×10^{-3} M; dinitrophenol (DNP) = 10^{-4} M. The results are presented as means ± S.E.; n, number of experiments.

"$P < 0.01$ compared with the corresponding J_{sm}.

**$P < 0.01$ compared with zero.

nsNot significantly different compared with condition a.

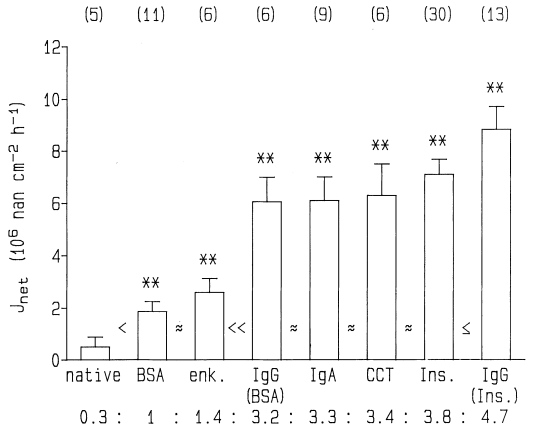

Fig. 3. Net fluxes of native or polypeptide-covered nanospheres (histograms) with the calculated relative values, the net flux of BSA-coated nanospheres being taken as reference. The histograms represent the mean ± S.E. (number of experiments in parentheses). $^{**}P < 0.01$ compared with zero; $\approx$, $\leq$, $<$, $<$, not significantly different, marginally significant, $P < 0.05$, $P < 0.01$ compared with the preceding histogram.

to $(25.5 \pm 2.0) \times 10^{-14}$ μmol/nan; $S_{0.5}$ ($= K_m$) is 94.8 ± 15.3 μM. Thus at the reference coating concentration of 6.5 μM, that we used for all polypeptides examined, the insulin adsorbed is only about 6% of the maximum adsorbable per nanosphere. Fig. 2 also shows the total insulin bound to all the nanospheres present (in μg) as a function of the insulin concentration in μg/ml; 2800 μg/ml is the maximal concentration of soluble bovine insulin which can be reached. At this concentration 420 μg insulin is adsorbed on all the nanospheres present, i.e. about 83% of the calculated maximal amount of adsorbable insulin (505 μg).

Third, in spite of the fact that at the reference coating concentration of 6.5 μM the adsorbed insulin is only 6% of the maximum adsorbable, if fluxes of nanospheres coated at the maximal reachable concentration of soluble insulin (4.7×10^{-4} M, i.e. 2800 μg/ml) are measured, a small, not significant increase of J_{ms} and J_{net} is obtained (Table 3d); J_{sm} does not change at all, as predicted. Thus net absorption is already maximal or almost maximal (70% of the maximum) at a coating concentration of 6.5×10^{-6} M, i.e. at a concentration much lower than that able to cover the nanosphere completely with insulin. It is noteworthy that CCT-cn also display about 70% of maximal transport, when coating occurs with 6.5×10^{-6} M carbocalcitonin. Thus it seems that the apparent affinity of receptors for insulin and CCT is very similar and a few molecules of these polypeptides adsorbed on the nanosphere are sufficient to offer the maximal binding and transport probability.

4. Discussion

Four main conclusions can be drawn from the results reported.

First, in agreement with and in further support of the hypothesis of antigen sampling [1,4,5], the transport system seems to accept many different polypeptides with very different molecular weights, besides CCT and ACTH; moreover, it also accepts them on nanoparticles, in agreement with vesicular transcytosis. The net absorption of coated nanoparticles is related to active transport as it is strongly affected by temperature variations ($Q_{10} = 2.2$) and completely abolished by metabolic inhibitors; this has also been observed for the net transport of free CCT not adsorbed on nanospheres [1].

Second, uncoated beads are not transported and transport selectivity is shown for the different polypeptides carried by nanospheres, in agreement with the previous conclusion that active transport should be supported by receptor-mediated endocytosis (RME) [2–4]. On the basis of the results reported in Table 1, Fig. 3 summarizes transport selectivity, reporting the net transports of the uncovered or polypeptide-covered nanospheres as histograms ordered according to their increasing values; the transport ratios, taking BSA-cn as a reference, are calculated and reported, as well as the statistical probability for the differences between transports. Besides the fact that uncovered beads do not exhibit any significant net flux, the figure shows that nanospheres completely covered with BSA display a significant but very small net flux. No increase in transport, compared with that of BSA-cn, is obtained with nanospheres coated with enkephalin+BSA, so it is doubtful that enkephalin can be recognized by receptors, at least better than BSA: either the sequence of enkephalin amino acids (Tyr-Gly-Gly-Phe-Leu) is not recognized by receptors, or it is recognized with the same affinity of sequences present in BSA, or the small enkephalin molecules are too deeply buried

among the larger BSA molecules, all adsorbed on the bead surface, so that much greater enkephalin concentrations in the covering medium would be needed to produce an effect of any extent. By contrast, net transport significantly increases by 3–4 times that of BSA-cn when the nanospheres are coated with anti-BSA IgG, IgA, CCT and insulin, and further increases to almost 5 times with anti-insulin IgG. In all these cases BSA was also adsorbed to block the uncovered surface sites. Considering that the analysis performed in the insulin case shows that only about 6% of the single nanosphere surface is covered by the tested polypeptide at the reference covering concentration of 6.5×10^{-6} M, it is clear that the majority of the bead surface is occupied by BSA and the whole increase in transport is related to a few molecules of the polypeptide tested. Even if the adsorbed molecules of the polypeptide are considerably increased (see the CCT case), even so as to cover the nanosphere completely (see insulin), nanosphere net transport displays only a modest increase. The conclusion is that, at the reference covering concentration, although the adsorbed molecules only cover a minimal area of the bead surface, the binding and transport probability are almost maximal. As for the tested antibodies, the coating and transport kinetics were not determined. However, at the reference covering concentration of 6.5×10^{-6} M, anti-BSA IgG-cn and IgA-cn exhibit net transports not significantly different from those of CCT-cn and insulin-cn, and anti-insulin IgG-cn only marginally larger. Thus the affinity of these antibodies for receptors should be equal to or smaller than but certainly not greater than that displayed by CCT or insulin. It is to emphasize that adsorption of the polypeptide on the nanoparticle forces the molecule into positions that do not necessarily represent the best orientation for easy binding to receptors. Hence the determined affinities for receptors (CCT, insulin) or those reasonably presumable (IgA, anti-BSA IgG, anti-insulin IgG) can only be the apparent affinities of the polypeptide adsorbed on the bead: these apparent affinities do not necessarily correspond to the actual affinities of the free polypeptides. That being said, it is worth noting that in rat intestinal mucosa, covering follicles of Peyer patches, nanoparticles of the same material and size, coated with the same human colostral IgA, are transepithelially transported with a ratio of 3.7 ± 1.3 ($n=7$) compared with nanoparticles coated with BSA only [9], in accordance with the present findings for nasal mucosa (ratio: 3.3).

Third, the kinetics of net CCT transport as a function of CCT concentration in the coating medium, and thus as a function of CCT molecules adsorbed per nanosphere, is sigmoidal, with a Hill number of 3.3. The probability that nanospheres are transported should be related to the probability that binding points are formed between nanospheres and receptors; the latter probability is proportional to the number of polypeptide molecules adsorbed on the nanosphere. Thus the kinetics should be hyperbolic. However, if the single nanosphere preferentially requires more than one binding point, to be forced and more firmly entrapped in the forming vesicle, and the settlement of one of them facilitates the further settlement of the other binding points, the kinetics should become sigmoidal. The Hill number obtained suggests that there are three cooperating binding sites per nanosphere. Alternatively, the data points in Fig. 1 can be interpolated by a hyperbola, however, intercepting the x-axis at a threshold concentration. In this case nanospheres would be transported in relation with the formation of only one or many, non-cooperating binding sites, but a threshold number of polypeptide molecules adsorbed on the nanosphere would be requested to reach a minimal binding probability.

Fourth, J_{sm} is independent of temperature and coating, or minimally dependent on coating. Thus J_{sm} seems to depend on a passive non-specific permeation. Considering bead dimension, it is unlikely that the nanosphere passive flux crosses intercellular junctions, especially so because it is not affected by regulation of actin cytoskeleton, which controls intercellular junctions [3]. J_{sm} should cross the epithelium through a few very large paracellular pores, much more similar to nanolesions of a negligible total area, so as not to short circuit the transepithelial electric potential difference.

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