Homogeneous Immunoassay Based on Chemiluminescence Energy Transfer

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The chemiluminescent compound aminobutyethyl-isoluminol (ABEI) and its isothoncyanate derivative have been coupled to a range of haptenes (progesterone, cyclic AMP, cyclic GMP) and protein antigens (IgG, C9). All the derivatives were chemiluminescent, immunologically active, and stable for more than six months. When the ABEI-labeled antigens bind to their respective fluorescein-labeled antibodies, there is a shift in the ratio of chemiluminescence at 460 nm (blue) to that at 525 nm (green). This nonradiative energy transfer was used to establish homogeneous immunoassays, which require no separation step. These assays were at least as sensitive as the conventional radioimmunoassays and could accurately measure substances in serum (i.e., for IgG, results correlated by \( r = 0.96 \) relative to those by \( { }^{125} \)I assay) and tissue extracts (cyclic AMP, \( r = 0.91 \) relative to \( { }^{3} \)H assay), and also were used to evaluate the kinetics of antibody-antigen binding. Chemiluminescence energy transfer provides a new method for quantifying ligand-ligand interactions in the \( 10^{-15} \) to \( 10^{-18} \) mol range without first separating bound and free ligand. This provides a unique opportunity to investigate chemical events in single cells and intact cells.

Additional Keyphrases: chemiluminescence energy transfer immunoassay - fluorescent-labeled antibody - radioimmunoassay compared - ligand-ligand interactions - kinetics of antigen-antibody binding - single-cell chemistry

Investigation of many biological problems requires a method for quantifying ligand-ligand interactions. Immunoassay, a technique based on the interaction between an antibody and an antigen, is a highly sensitive and specific method for measuring a wide range of substances in biological and clinical interest (1). Because of its high sensitivity of detection, \( { }^{125} \)I has been the most popular label used in this procedure. However, radioisotopes such as \( { }^{125} \)I suffer from several disadvantages (2, 3). They can be hazardous; the shelf life of the reagents is usually only a few weeks; and high sensitivity often requires long counting times. Moreover, homogeneous assays, with no separation step, are not possible with radioisotopes.

Chemiluminescence, the emission of light from a chemical reaction, can be exploited as a highly sensitive method for measuring substances of biological interest (2–4). Detection of some substances in the \( 10^{-18} \) to \( 10^{-21} \) mol range is possible. The covalent coupling of certain synthetic chemiluminescent compounds—such as luminal, isoluminol, and acridinium ester derivatives—to antigens and antibodies has enabled development of immunoassays of comparable or better sensitivity than those involving radioactive labels (5–11). These chemiluminescent labels are stable—apparently indefinitely—on storage, and can be detected within a few seconds of initiation of the chemiluminescence.

Recently we reported (12) that the wavelength of the emitted light shifts when chemiluminescence-labeled (with aminobutyethyl-isoluminol, ABEI) IgG or cyclic AMP bind to their respective fluorescein-labeled antibodies. Here we report that this phenomenon of nonradioactive energy transfer occurs with antigens having a wide range of relative molecular mass (\( M_r \), 314–150 000) and can be used as a homogeneous immunoassay procedure for measuring substances in biological samples.

Materials and Methods

Apparatus

Chemiluminescence was detected in a specially constructed lumimeter (13), in which a highly sensitive low-dark-current photomultiplier tube (type P-8320B, Twentieth Century Electronics, Croydon, U.K.; or type 97447AM, EMI Electronics, Uxbridge, U.K.) is coupled to a high-voltage (900–1200 V) supply, scalar, and a square-wave discriminator. The photomultiplier was in a cooled (–20°C), light-tight housing, the output connected to an eight-digit scalar and interfaced to an LSI 11 Digital Equipment computer (RT11 operating system) plus dual floppy disc drive. Energy transfer was detected with a specially constructed dual-wavelength lumimeter (Figure 1) containing two sets of photomultiplier tubes, high-voltage supplies, scalars, transient recorders, and oscilloscopes. Light emission was monitored at two wavelengths simultaneously by placing thin-film interference filters (type B40, with a half-bandwidth of 7 nm; Balzers Ltd., Berkhamshead, Herts., U.K.) in front of each photomultiplier. To evaluate the apparatus, we compared the ratio of light emission at 470 and 510 nm from a Ca²⁺-activated photoprotein, obelin, from the hydroid Obelia geniculata (14).

Processes

Synthesis of chemiluminescent-labeled antigens. ABEI was synthesized from 4-nitrophthalic acid with an overall yield of 2%, by the method of Schroeder et al. (5). ABEI isothoncyanate (ABEI-NCS, Figure 2) was prepared from ABEI and thiophene by the method of Britzinger et al. (9, 15). ABEI-NCS appeared as a single blue-fluorescent spot after thin-layer chromatography on silica gel in a solvent system of chloroform/95% ethanol (7/3, by vol). Its \( R_f \) was 0.75, compared with 0.06 for ABEI. We determined the \( M_r \) of ABEI-NCS to be 318.2 by electron-impact mass spectrometry (theoretical \( M_r = 318.2 \)). To label proteins with ABEI, we coupled them with ABEI-NCS in Na₂CO₃/NaHCO₃ buffer, 0.1 mol/L, pH 9.5 (12). ABEI-labeled succinyl cyclic AMP (ABEI-scAMP, Figure 2) and cyclic GMP (ABEI-}

2 Nonstandard abbreviations: ABEI, aminobutyethyl-isoluminol; ABEI-scAMP, ABEI-scGMP, and ABEI-sGMP, ABEI-labeled succinyl cyclic AMP, succinyl cyclic GMP, and succinyl progesterone, respectively; ABEI-NCS, ABEI isothoncyanate; IgG, immunoglobulin G.
scGMP) were synthesized by a mixed anhydride reaction and purified by thin-layer chromatography on cellulose, with butanol/acetic acid/H2O (60/15/25, by vol) as solvent (Rf ABEI-scAMP = 0.53; Rf ABEI-scGMP = 0.48; Rf ABEI = 0.64) (12). ABEI-labeled succinyl progesterone (ABEI-sP; Figure 2) was prepared from the N-hydroxysuccinimide ester of progesterone-11α-hemisuccinate by the method of Kohen et al. (16).

Fluorescent antibodies. Fluorescein-labeled anti-IgG was prepared by using fluorescein isothiocyanate in the Na2CO3/NaHCO3 buffer, then purified by gel filtration (12). The mean number of fluoresceins per molecule of IgG ranged from four to 12 in different preparations.

Assay for ABEI chemiluminescence. We assayed ABEI at pH 7.4, 9, or 13, using microperoxidase (MP 11; Sigma Chemical Co.) and H2O2 as previously described (9, 12, 17). At pH 9, ABEI produced 4.2 × 1018 luminescence counts per mole in 10 s, with a background count of 2000 (SD 60), resulting in a detection limit for ABEI of about 5 × 10−17 mol.

Results
Characterization of Chemiluminescent-Labeled Antigens
ABEI-labeled antigens were characterized for purity, stability, chemiluminescent activity, and immunological activity in a conventional radioimmunoassay. There was no detectable free ABEI in any of the preparations, which were stable on storage at −20 °C for at least six months. Measurement of ABEI in the ABEI-labeled proteins by spectrophotometry (ε314 = 12 000 L·mol⁻¹·cm⁻¹) and by chemiluminescence showed 0.2–3 mol of ABEI per mole of protein with no apparent change in quantum yield—unlike luminol, the quantum yield of which is drastically reduced when coupled through its amino group. ABEI-scAMP and ABEI-sP showed no change in quantum yield at pH 13, but light yield was three- to fivefold less when measured at pH 9. There was no detectable change in quantum yield or kinetics when the ABEI-labeled antigens bound to their respective antibodies, in contrast to the enhanced chemiluminescence reported for ABEI-sP bound to some antibodies (18).

ABEI-labeled rabbit IgG or C9 showed no loss of immunological activity as assayed by a conventional 125I radioimmunoassay (17, 19). ABEI-scAMP had up to twice the affinity of cyclic AMP for anti-cyclic AMP antibodies, and ABEI-sP had sevenfold more affinity than progesterone for antibodies to progesterone.

These results confirmed that the ABEI-labeled antigens were both chemiluminescent and immunologically active. Therefore, we established standard curves for rabbit IgG (range, 6.7 fmol–67 pmol), human complement component C9 (10 nmol–10 μmol), cyclic AMP (50 fmol–50 pmol), and progesterone (47 fmol–47 pmol) from data obtained by using the ABEI-labeled antigens and cellulose-bound anti-IgG as immunoabsorbent (20).

Chemiluminescence Energy Transfer
To establish whether energy could be transferred between the ABEI-labeled antigens and their respective fluorescein-
labeled antibodies, we incubated these together at pH 7.4 under conditions favoring >95% binding of the antigen. Chemiluminescence was triggered at pH 9, a pH at which antibody–antigen binding is unaffected. We compared the ratio of light emission at 460 (blue)/525 (green) nm for ABEI-labeled antigen in the presence of unlabeled IgG or fluorescein-labeled nonimmune IgG (Figure 3). Except for cyclic GMP, with our present apparatus (uncorrected for geometry and the spectral sensitivity of the photomultiplier tubes), the ratio of luminescence for ABEI at 460/525 nm shifted from about 4, for unlabeled IgG and fluorescein-labeled nonimmune IgG, down to 1 to 2 when fluorescein-labeled immune IgG was used. The reason for the higher values of 2.98 for fluorescein-labeled antibodies to cyclic GMP is unknown.

This shift in wavelength of the light emitted by ABEI-labeled antigen when bound to fluorescein-labeled antibody

Fig. 3. Chemiluminescence energy transfer
Chemiluminescent (ABEI-labeled) antigens were incubated with fluorescein-labeled or unlabeled antibodies or fluorescein-labeled nonimmune IgG for 2 h at room temperature (20°C) in a final volume of 100 μL. Chemiluminescence was then activated by adding 100 μL of microperoxidase (5 μmol/L of sodium barbitol, 100 mmol/L, pH 9, containing 100 mg of bovine serum albumin per liter), followed by 20 μL of H2O2 (175 mmol/L) from a spring-loaded syringe. Light emission was monitored at 460 nm and 525 nm simultaneously, and the ratio calculated. Results are the mean ± SD of three observations.

![Graph](image)

Fig. 4. Homogeneous immunoassay for progesterone
(i) equilibrium assay: 50 μL of ABEI-αP (2.9 nmol/L of sodium phosphate, 50 mmol/L, pH 7.4, containing 100 mg of bovine serum albumin per liter) was incubated overnight at 4°C with 25 μL of progesterone standards (3.5 mmol/L to 35 μmol/L plus 25 μL of 2400-fold diluted fluorescein-labeled anti-progesterone IgG (approx 50% binding));
(ii) non-equilibrium assay: progesterone standards were incubated with fluorescein-labeled anti-progesterone IgG overnight at 4°C, then the ABEI-αP was added and the suspension incubated at room temperature for a further 30 min. Chemiluminescence was initiated as described in Fig. 3, and the ratio of light emission at 460 and 525 nm was monitored. Results represent the mean ± SD of three determinations.

Fig. 5. Association and dissociation of antibody–antigen complex, as studied by chemiluminescence energy transfer
At room temperature (20°C), 1.5 mL of ABEI-αcAMP, 50 nmol/L, was incubated with 0.75 mL of fluorescein-labeled anti-cyclic AMP IgG (100 μg of IgG per milliliter) in sodium phosphate, 50 mmol/L, pH 7.4. At various times 75-μL aliquots were removed, and the chemiluminescence at 460 and 525 nm was measured at pH 9 immediately, as described for Fig. 3. After 1 h, 0.4 mL of cyclic AMP (1 mmol/L) was added to 1 mL of the remaining solution (indicated by arrow), and 100-μL aliquots were then assayed for chemiluminescence at 460 and 525 nm. Results shown are the average of two determinations.

![Graph](image)

Biological Samples

With the homogeneous chemiluminescence energy transfer immunoassay, we could measure cyclic AMP extracted from pigeon erythrocytes (21) and IgG in samples of rabbit serum. These measurements (γ) correlated well with results by conventional radioimmunoassay (cyclic AMP: y = 1.18x + 0.68, r = 0.91, n = 17; IgG: y = 1.01x – 0.23, r = 0.96, n = 13).

Kinetics of Antibody–Antigen Binding

The association and dissociation of ABEI-labeled antigens with fluorescein-labeled antibodies could also be detected by chemiluminescence energy transfer (Figure 5), without the need to separate bound and free antigen. We could perform these studies over a pH range of 7 to 9 and a temperature range of 0 to 37°C.

Discussion

Nonradiative energy transfer occurs through dipole–dipole resonance between an excited donor molecule and an acceptor molecule. The result is a “red” shift in the light emitted, compared with that emitted from the donor alone. Sometimes changes in quantum yield and kinetics are also observed. The equations predicting the rate and efficiency of this phenomenon were first derived from Förster (22, 23):

\[ \text{Rate of energy transfer} = d^{-6} K^2 J_{a} n^{-4} k_{r} \times 8.71 \times 10^{23} \, \text{s}^{-1} \]

\[ \text{Efficiency of energy transfer} = d^{-6}/(d^{-6} + R_{0}^{-6}) \]

\[ R_{0} = (J K^2 Q_0 n^{-4})^{1/6} \times 97 \times 10^5 \, \text{nm} \]

where \( d \) is the distance between the centers of the chemiluminescent donor and fluorescent acceptor molecules; \( K^2 = \)
the orientation factor for dipole–dipole interactions; \( J \) = the spectral overlap integral; \( n \) = the refractive index of the medium between the donor and the acceptor; \( k_F \) = the rate constant of the emission by the donor; and \( Q_0 \) = quantum yield of the chemiluminescent energy donor in the absence of the acceptor. Fluorescence energy transfer has been used as a spectroscopic ruler in several biological systems (24, 25). In general, the donor and acceptor molecules should be within 5 to 10 nm to give 20 to 100% efficiency of transfer (24). The Stokes radius of an IgG molecule (\( M_r 150,000 \)) is about 4 nm. Because antibodies are divalent and the fluorescent antibodies used in these experiments contained a mean of four to 12 fluoresceins per molecule of IgG, the conditions for Förster-type energy transfer should be satisfied between an ABEL-labeled antigen and fluorescein-labeled antibodies. The results reviewed here are consistent with singlet–singlet Förster-type nonradiative energy transfer. Under the present experimental conditions, this should only occur within the antibody–antigen complex, because the concentration of either the antigen or the antibody would have to be 1–10 mmol/L for energy transfer to occur between the free molecules. This is \( 10^2 \) to \( 10^6 \) times the concentration of the reagents used (Figures 3–5). Intramolecular chemiluminescence energy transfer has been observed with phthalazine diones (26–29), whereas intermolecular chemiluminescence energy transfer has apparently been observed in some luminous coelenterates such as Aequorea, Obelia, and Renilla (30–33) and may occur in some red-emitting fish such as Malacosteus (34).

The decrease in light emission at 460 nm, concomitant with an increase at 525 nm when ABEL-labeled antigens bind to their respective fluorescein-labeled antibodies (Figure 3), provides the first demonstration of intermolecular chemiluminescence energy transfer within an antibody–antigen complex. The resulting homogeneous immunoassay (Figure 4) (12), which does not require a separation step, appeared to be considerably more sensitive than reported for either fluorescence energy transfer immunosassay (35) or the homogeneous enzyme (EMT®) immunosassay (36). Furthermore, the present assays could accurately measure substances in biological extracts (12) and the kinetics of antibody–antigen binding (Figure 5).

Thus far, our attempts to improve the magnitude of the energy transfer by using different chemiluminescent donors or fluorescent acceptors have failed. Little or no shift in wavelength occurs between ABEL-labeled rabbit IgG and sheep anti-rabbit IgG labeled with either rhodamine or 7-nitrobenzo-2-oxa-1,3-diazole. Nor has any energy transfer yet been detected between acridinium-labeled IgG—acridinium esters being good nonsotopic chemiluminescent labels (10, 11)—and fluorescein-labeled antibodies. Apparently, during the chemiluminescent reaction the excited \( N \)-methyl acridone is cleaved from the antigen; furthermore, this excitation reaction is best catalyzed at high \( \rho H \), which would disrupt the antibody–antigen complex. Oxalate esters are also unlikely to be good donors: these chemiluminescent compounds transfer energy via electron transfer (29), a process that only occurs when the donor and acceptor are within 0.2 nanometers of each other.

Chemiluminescence energy transfer therefore provides a highly sensitive method for quantifying ligand–ligand interactions without a separation step. The method is applicable not only to clinical samples (free hormone and drug measurement, and hormone-receptor binding studies), but also to the study of low-affinity, fast-dissociating ligand–ligand interactions, which is not possible in an assay requiring the separating of bound ligand from free. The method may also be useful in screening for monoclonal antibodies. Another exciting possibility is the use of chemiluminescence energy transfer for studying chemical events in intact cells and single cells, thereby circumventing two of the major limitations in the conventional biochemical approach (5, 27–39) and enabling the development of a new approach to cell activation and cell injury.

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